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Multitrophic Interactions in Soil

Editors:

Richard A. Sikora, Simon Gowen,
Rüdiger Hauschild & Sebastian Kiewnick

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Working Group
"Multitrophic Interactions in Soil and Integrated Control"

Proceedings of the WG Meeting

Multitrophic Interactions in Soil and Integrated Control

at Bad Honnef, Germany, June 1-4, 2003

combined with selected papers from the meetings

Thinking in Lines – From Research to Market Products

in Einsiedeln, Switzerland, November 2-4, 2000

and

Biological Mechanisms Affecting Nematode Management

in Reading, England, September 5-6, 2001

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Introduction

In the introduction to the bulletin published after the first study group meeting on *Tri-trophic interactions in the rhizosphere and root health* I stated that the participants were unanimous in their opinion that a multidisciplinary working group, designed to bring researchers from different fields together, should be organized at regular intervals. The IOBC general council agreed after seeing the results of that first gathering in Bonn. This meeting is the result of their foresight and support.

The bulletin you have in your hands right now, contains the papers presented at the first official meeting of the Working Group - *Multitrophic interactions in soil and integrated control*. I hope I speak for all those who attended this inaugural meeting, when I state it was an overwhelming success. The meeting was attended by 63 participants from 11 countries, with a large proportion of young scientists actively involved.

More importantly, the goal was attained of having a meeting with scientists from many different disciplines - bacteriology, mycology, nematology and entomology. In addition to scientists representing specific pests and diseases we also were able to attract researchers with very different approaches to biological control ranging from classical biocontrol over molecular biology to industrial applications.

The sessions were designed to stimulate interaction between these disciplines and not to isolate them into homogenous topics by pest or disease. This led to lively presentations, intense debate and constructive criticism. I believe everyone went home with a new view of the holistic nature of the soil ecosystem and the intricate interactions occurring in this environment. In addition, the meeting exposed those attending to many new methodologies for working with soil-borne biocontrol organisms and problems of commercialisation.

Evolution of the working group is also a part of the story reproduced in this bulletin. It contains research and concept papers presented at two earlier meetings. *Thinking in Lines – From Research to Market Products* in Einsiedeln, Switzerland, November 2-4, 2000 organized by Juerg Grunder and *Biological Mechanisms Affecting Nematode Management* in Reading, England, September 5-6, 2001 organized by Simon Gowen.

The papers from these meetings are included here because they deal with multitrophic interactions and they formed the basis for the fusion of smaller groups working in the soil ecosystem into this team which we believe will expand our knowledge and make further progress in integrated control in soil. I want to thank all those who contributed to the success of these three meetings and those who supported the evolution of this unique working group. In particular I want to thank Brain Kerry for his strong support.

Let me wish you all success in understanding and using microorganisms for integrated control of pests and diseases in the soil ecosystem.

Richard A. Sikora
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Contents

* papers pprepsented at the Einsiedeln-meeting, Switzerland, 2000;
** papers pprepsented at the Reading-meeting, England, 2001

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Multitrophic Interactions in Soil and Integrated Control
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Investigation of three nematophagous fungi in two potato cyst nematode suppressive soils

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Abstract: Two PCN suppressive soils, one from Ely and the other from Spalding in the UK, were investigated for the presence of three nematophagous fungi, *Pochonia chlamydosporia*, *Plectosphaerella cucumerina* and *Paecilomyces lilacinus*, using a range of physical and molecular techniques. *Pochonia chlamydosporia* was not detected in either soil using serial dilution on semi-selective media and PCR detection using specific primers. *Plectosphaerella cucumerina* was only detected in soil taken from Spalding and was estimated at around 700 CFU g^{-1} soil using serial dilution on semi-selective media, but its presence was confirmed in both soils by PCR analysis. *Paecilomyces lilacinus* was also only detected in soil taken from Spalding and was estimated to be at 2449 CFU g^{-1} soil. Each soil was baited with *Globodera pallida* cysts, after two weeks the baits were removed and plated onto water agar plates. Fifty infected eggs were removed and individually incubated in Czapex Dox liquid media. DNA was extracted and fungal isolates were identified by PCR analysis. None of the fungi taken from both soils were identified as *P. chlamydosporia* or *P. lilacinus*, 8 % and 28 % of the isolates taken from the baits added to the Ely and Spalding soils respectively were identified as *P. cucumerina.*

Keywords: PCN, PCR, biological control agents, semi-selective media, baiting, *Verticillium chlamydosporium*

Introduction

The potato cyst nematodes (PCN), *Globodera rostochiensis* (Wollenweber) and *G. pallida* (Stone) are serious pests in potato production causing yield losses valued annually at 300 M ECU in the European Community (Mulholland *et al.*, 1996). Growers are encouraged to adopt an integrated approach to PCN management using resistant cultivars and nematicides, in addition to crop rotation (Haydock & Evans, 1998). The development of fungal biological control agents to manage nematodes may become an important alternative to chemical control in agriculture, especially as restrictions on the use of some leading nematicides has occurred in some European countries.

Understanding the factors that cause inconsistencies in the biological control of nematodes by nematophagous fungi has been hampered by a lack of useful techniques to determine the presence and abundance of these fungi in the rhizosphere and soil. Also, it is important for regulatory purposes to develop methods to monitor the specific fungal biological control agents after their release (Avis *et al.*, 2001). A range of techniques, both physical and molecular, are now available to monitor specific fungi in the environment. Techniques have been combined to monitor the nematophagous fungus *Pochonia chlamydosporia* var. *chlamydosporia* (Goddard) Zare and Gams (Zare *et al.*, 2001) (until recently classified as *Verticillium chlamydosporium* Goddard) in the rhizosphere and bulk soil (Hirsch *et al.*, 2001), to improve understanding of the tri-trophic interactions between the plant, fungus and nematode pest in the rhizosphere.

Semi-selective media have been developed for a range of fungi (Mitchell *et al.*, 1987; Kerry *et al.*, 1993; Steadman *et al.*, 1994; Atkins *et al.*, 2001), and these enable an estimate of the abundance of fungi in soil and root samples. Although dilution plate methods have significant limitations, the use of selective media has enabled the estimation of relative changes in abundance and greatly increased the understanding of the ecology of selected nematophagous fungi in soil (Kerry, 2000). PCR-based methods to detect mycorrhizal and pathogenic fungi in plant roots have been reported (Gardes *et al.*, 1991; Lovic *et al.*, 1995). These have relied on primers to recognise specific sequences within the mitochondrial genome, or the transcribed (ITS) and non-transcribed (IGS) spacers within the ribosomal RNA genes (White *et al.*, 1990). Recently, PCR detection of *P. chlamydosporia*, was achieved using primers based on the β-tubulin gene (Hirsch *et al.*, 2000). Baiting techniques (Rodriquez-Kabana *et al.*, 1994; Sikora *et al.*, 1994) have enabled the pathogenic capability of isolates to be assessed *in-situ*.

Here, the use of a range of techniques both physical and molecular are reported to detect, quantify and assess the pathogenic ability of three nematophagous fungi that have shown biological control ability towards PCN (Jacobs, 2000) in two PCN suppressive soils in the UK.

Material and methods

Cultures

Three nematophagous fungi, *Plectosphaerella cucumerina*, *Paecilomyces lilacinus* and *Pochonia chlamydosporia*, were isolated from PCN cysts taken from potato fields on the island of Jersey. Cultures of the fungi were maintained on potato dextrose agar (PDA-Oxoid, UK) plates at 25 °C and stored at 4 °C on PDA plates, or at –80 °C in 15 % glycerol stocks.

Soil samples

Soil was collected from two farms in the UK reported to have PCN suppressive soil. One site was in Ely, Cambridgeshire (A. D. Barker personal communication) and the other in Spalding, Lincolnshire (Crump, 1998). Soil was stored at 4 °C.

Detection of P. chlamydosporia, P. cucumerina *and* P. lilacinus *in PCN suppressive soil using semi-selective media*

The presence of each fungus was detected by preparing a serial dilution series using 1 g of pooled soil from a thoroughly mixed sample from each site. Aliquots (0.2 ml) of dilution (10^{-2} - 10^{-4}) were spread onto the semi-selective medium for *P. cucumerina* (Atkins *et al.*, 2001), *P. chlamydosporia* (Kerry *et al.*, 1993) and *P. lilacinus*, modified from media described by Mitchell *et al.*, 1987. The media did not contain benomyl, as the isolates used were sensitive to the fungicide, and the level of PNCB (Pentachlornitrobenzene 99%, Pentachlornitrobenzol 99%, Aldrich) was reduced to 28 mg. The modified medium was prepared by combining 10 g sodium chloride, 28 mg PCNB, 39 g potato dextrose agar (PDA, Oxoid) and high quality water (de-mineralised and deionised) to bring the volume to 1 litre. Plates were incubated at 25 °C and colonies counted after 3 and 11 days.

Detection of P. chlamydosporia *and* P. cucumerina *in PCN suppressive soil using PCR*

DNA was extracted from four replicate 0.25 g samples of pooled soil, which had previously been sieved and thoroughly mixed, from each of the soils using a soil DNA extraction kit (Mo-Bio Laboratories, UK), and an aliquot (1 µl) from each replicate was used in a PCR reaction. Primers and reaction conditions for detection of *P. cucumerina* and *P. chlamydosporia* are described by Atkins *et al.* (2001) and Hirsch *et al.* (2000) respectively.

PCR products were compared to PCR fragments generated from positive control DNA extracted from pure culture.

Baiting PCN suppressive soils

Soil, from each site, was sieved through a 5 mm sieve and 1 kg put into a 12 cm diameter pot. Treatments were duplicated. The soil was baited with 25 *G. pallida* cysts placed inside a nylon mesh of 60 µm pore size (Lockertex, UK) and held inside a slide mount (Gepe, Sweden) as described by Sikora *et al.*, (1994). The slide mounts were pushed just below the surface of the soil and the pots were incubated at 21 °C for two weeks with light watering every two days. Baits were collected and the cysts opened using a cyst crusher. Eggs were then plated out onto water agar containing antibiotics (0.5 g technical agar, 50 mg streptomycin, 50 mg chloramphenicol, 50 mg chlortetracycline per litre) and incubated at 25°C for two days. Baits were added to a control of sterilised sandy loam soil and incubated as above. Infected eggs were counted after two days and the percentage infection calculated. The proportion of eggs infected from each soil was compared to the control and analysed using one way analysis of variance (ANOVA) using the Genstat programme (Genstat 5 Committee, 1993).

ERIC fingerprinting

DNA was extracted from a pure culture of each fungus using a method described by Arora *et al.*, (1996). DNA was amplified in a PCR reaction using ERIC (Enterobacterial Repetitive Intergenic Consensus sequences) primers described by White *et al.*, (1990). The conditions for amplification as described by Arora *et al.*, (1996) were modified to make the reaction less stringent. The modifications were as follows: primer concentrations were reduced from 50 pM to 10 pM, magnesium chloride concentrations were increased from 1.5 mM to 2.5 mM. The amplification conditions were changed to 30 cycles of 94 °C for 1 minute, 46 °C for 1 minute and 72 °C for 1 minute, followed by a final DNA extension time of 72 °C for 5 minutes. PCR products were separated on 2 % agarose gels in TBE buffer (0.089 M Tris-HCl pH 8.6, 0.089 M boric acid, 0.002 M EDTA) stained with ethidium bromide (0.5 µg ml^{-1} H_2O).

Identification of* P. lilacinus, P. cucumerina *and* P. chlamydosporia *from infected PCN eggs using PCR

Cysts were removed from a 200g sample of soil taken from each site using a Fenwick can. Cysts were crushed and plated out as above. Twenty infected eggs (infected eggs were classified as eggs with mycelia growing from them) were taken from each soil, and individual eggs were each added to 10 ml Czapex Dox liquid medium and incubated at 25 °C for two weeks. DNA was extracted from the fungal culture that developed from each infected egg using an alkaline lysis method (Klimyuk *et al.*, 1993). DNA was amplified using specific primers for detection of *P. chlamydosporia* (Hirsch *et al.*, 2000), *P. cucumerina* (Atkins *et al.*, 2001) and the semi-selective primers for *P. lilacinus* (forward primer: CAA GAC TGT CAG CGC TCT GTC: reverse primer: CTG CGC GAC TTG GAG TCC TGA A), and compared to DNA amplified from a pure culture of each fungus. Conditions for PCR amplification of *P. lilacinus* DNA were as follows: PCR reactions of 20 µl contained 0.1 µM each primer, 2 µl x10 PCR reaction buffer (Roche UK, 1.5 mM Mg^{2+}), 0.1 µM each dNTP, 1 U *Taq* polymerase (Roche), 1 µl DNA (20-60 ng). PCR conditions were optimised as follows: 95 °C followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, with a final

incubation at 72 °C for 5 min. Fifty infected eggs were taken from each bait experiment and incubated as above.

Results

Pochonia chlamydosporia was not detected at either field site, when soil was plated onto the semi-selective medium. For *P. cucumerina* the number of fungal propagules in the Spalding soil was estimated to be 700 CFU g^{-1} soil, whereas, no colonies were detected on the semi-selective medium from plates inoculated with soil from Ely. *Paecilomyces lilacinus* was estimated at Spalding at 2449 CFU g^{-1} soil, but was not present in the Ely site.

The presence or absence of either *P. chlamydosporia* and *P. cucumerina* was confirmed by PCR using selective primers for each fungus. *Pochonia chlamydosporia* was not detected in either soil, whereas *P. cucumerina* was detected in all four replicates taken from Spalding, and in two of the replicates taken from the Ely soil (Figure 1). The primers for detection of *P. lilacinus* were designed from the serine protease gene from the fungus (Accession number l29262). When tested against a range of other fungi they cross reacted with *Metarhizium anisopliae*, therefore, these primers could not be used to detect the fungus from mixed cultures such as soil, but did allow detection of *P. lilacinus* from pure culture such as the fungi extracted from infected PCN eggs.

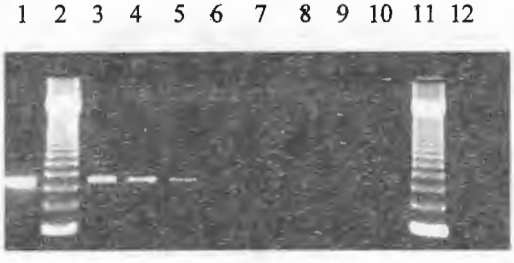

Figure 1.Investigation of *P. cucumerina* in soil using selective PCR primers. Lanes 2 and 11 = 123 bp maker; lane 1 positive control with primers for *P. cucumerina*; lanes 3 to 6 replicate detection of *P. cucumerina* in Spalding soil; lanes 7 to 10 replicate detection of *P. cucumerina* in Ely soil; lane 12 negative control

The use of the ERIC fingerprints gave a distinctive band profile for the three isolates taken from Jersey (Figure 2). The use of the selective primers for *P. chlamydosporia* and *P. cucumerina*, and the semi-selective primers for *P. lilacinus* were able to differentiate between the fungi (Figure 3), and could be used successfully to detect the presence of the fungus from infected nematode eggs.

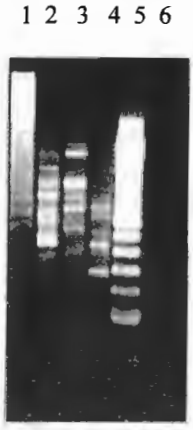

Figure 2. ERIC fingerprint profile of: lane 2, *P. cucumerina*; lane 3, *P. lilacinus*; lane 4, *P. chlamydosporia*; lane 1 = 100 bp marker; lane 5 = 123 bp marker; lane 6 = negative control

Figure 3. Detection of nematophagous fungi from pure culture using selective and semi-selective PCR primers: detection of: Lane 1, *P. chlamydosporia*; Lane 2, *P. cucumerina*; Lane 3, *P. lilacinus*: Lane 4 = 123 bp marker; Lane 5 = negative control

None of the fungi was detected using PCR, in the 20 infected eggs taken from both sites. The proportion of infected eggs in the baited cysts added to the soil from Ely was 15 % (± 1.9 n=8), and in the soil from Spalding the level of infection eggs was similar at 14 % (± 1.6 n=8). Both these results were significantly greater than the 9 % (± 1.09 n =6) (with F pr of 0.015 (SED 2.7) and 0.021 (SED 2.74) respectively) infection of eggs observed in the control. Of the 50 infected eggs taken from the Ely baits four colonies (8 %) were identified by PCR as *P. cucumerina*, whereas, 14 colonies (28 %) were identified as *P. cucumerina* from the Spalding soil. None of the fungi taken from the infected eggs were confirmed by PCR analysis to be *P. lilacinus* or *P. chlamydosporia* (Table 1*)*. Fungi that infected the control eggs were not identified.

Table 1. Summary of results for detection of *P. cucumerina*, *P. lilacinus* and *P. chlamydosporia* from soil taken from two PCN suppressive sites (Ely and Spalding) using dilution plating, PCR detection and baiting of soil. (NT= not tested)

Soil	Fungus	Detection of fungus by: Dilution plate	PCR of soil DNA	Baiting (Egg infection %)
Ely	*P. cucumerina*	-	±	8
	P. lilacinus	-	NT	-
	P. chlamydosporia	-	-	-
Spalding	*P. cucumerina*	700	+	28
	P. lilacinus	2449	NT	-
	P. chlamydosporia	-	-	-

Discussion

The three nematophagous fungi investigated have shown potential as biological control agents against PCN (Jacobs, 2000). In this report we have investigated the presence of all three fungi in two PCN suppressive soils using a variety of techniques. PCR techniques using ERIC primers have been used to generate specific fingerprint profiles of isolates that distinguish them from other fungi. Serial dilution, and plate counts can provide an estimation of the levels of the three fungi within the soil, but there are limitations to this technique, as the CFU originating from spores and hyphae cannot be separated, which makes interpretation of the data difficult. PCR analysis of the soil was more sensitive than the serial dilution method with the presence of *P. cucumerina* being detected in 50 % of the samples taken from the Ely site, although it confirmed that *Pochonia chlamydosporia* was undetectable in either soil.

Although PCR enables a quick and informative analysis of the soil samples it is limited in that it is not quantitative, nor does it provide information of the physiological state of the fungus within the soil. A more accurate method of assessing the pathogenicity of the fungi within the soil was provided by the baiting method. This allowed PCN pathogenic fungi to be readily isolated, and when combined with the PCR identification method, allowed a rapid screening and identification method for the fungi. Surprisingly, *P. lilacinus* was not observed infecting the PCN bait, even though it was by far the most abundant of the three fungi investigated in the soil taken from Spalding. *Plectosphaerella cucumerina*, although detected at a very low densities within the soil was found to infect a significant proportion of the eggs in the bait. In this report we have not identified the reason why these soils were suppressive to PCN, but we have demonstrated that *P. cucumerina* may play a role in the field site at Spalding. The results highlight the importance of assessing interactions between biological control agents, their hosts, and the environment, whether in the field (as in this case), or under experimental conditions such as pot tests.

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Fungal molecular diagnostics of nematophagous fungi

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Abstract: The ability to identify accurately an organism is fundamental to all aspects of fungal diagnostics and epidemiology whether this is in the field of plant pathology, medical science, environmental studies or biological control. Conventional methods have often relied on identification of disease symptoms, isolation and culturing of organisms, and laboratory identification by morphology and biochemical tests. These methods rarely allow diagnosis at the sub-specific level and increasingly there is a need to identify the role of individual isolates or strains in an interaction, which is possible using molecular techniques. There is an increasing move towards molecular diagnostics of fungi in all fields. Since its introduction in the mid 1980's PCR has provided many molecular diagnostic tools, and is readily applied to the monitoring and identification of fungi from environmental samples. Here, the use of specific PCR primers to identify nematophagous fungi from samples of soil, roots and even individual infected nematode eggs, arbitrary PCR primers to track released isolates to monitor efficacy, and real-time PCR to quantify a nematophagous fungus from soil are discussed. This technology is combined with more conventional means of fungal diagnostics such as serial dilution onto selective media and baiting to provide complementary data of fungal interactions in the environment and the potential of selected fungal biological control agents.

Key words: PCR diagnostics, nematophagous fungi, real-time PCR

Introduction

In phytopathology early identification of the causative agent of disease is paramount in order to recognise the pathogen, and implement regulations involving control and quarantine. Conventional methods often rely on identification of disease symptoms, isolation and culturing of organisms, and laboratory identification by morphology and biochemical tests. These methods, although the cornerstone of fungal diagnostics, can lead to problems in identification, resulting in incorrect interpretation, diagnosis and ultimately, treatment. The methods rely on experienced, skilled laboratory staff, the ability of the organism to be cultured and are time consuming, non-quantitative, prone to contamination and error and in the case of plant and medical pathology often delay treatment.

PCR Diagnostics

New, rapid screening methods are being developed and increasingly used in all aspects of fungal diagnostics. These methods include immunological methods, DNA/RNA probe technology and polymerase chain reaction (PCR) technology. How these methods have been implemented in plant pathology has been intensively reviewed, most recently by McCartney *et al.*, 2003, and more generally by Atkins and Clark, 2003. The past decade has seen many advances in fungal molecular diagnostics, the most rapid being in PCR technology. In contrast to more conventional methods such as serial plate dilutions, samples can be tested directly, and isolates do not require culturing. They are rapid, highly specific and can be used to detect minute quantities of fungal DNA from samples before symptoms occur, therefore,

allowing implementation of early control methods. With improved DNA extraction methods, and with adequate controls, PCRs can be run routinely and do not require a high level of expertise for interpretation of the results. Specific primers have been developed for the identification of four major nematophagous fungi, *Pochonia chlamydosporia* var. *chlamydosporia*, (Hirsch *et al.*, 2000), *Pochonia chlamydosporia* var. *catenulata* (Atkins *et al.*, 2003A), *Plectosphaerella cucumerina* (Atkins *et al.*, 2003B) and *Paecilomyces lilacinus* (Atkins *et al.*, 2003C). These primers will prove invaluable in the monitoring of the fungi in trials to investigate their ability to control nematode pests and can be used routinely to identify the fungi from soil, roots and nematode eggs. The use of arbitrary primers can be used to further discriminate the released isolate from other indigenous isolates, therefore, provides the ability to assess individual isolates *in situ*. A number of primers exist for this such as RAPDs (random amplified polymorphic DNA: Welsh and McClelland, 1990), REPs (repetitive extragenic palindromic: Higgins *et al.*, 1982) and ERICS (enterobacterial intergenic consensus sequences: De Bruijn, 1992; Arora *et al.*, 1996). Interpretation and review of these methods is outlined by Bridge and Arora (1998).

Quantification of PCR

One of the main limitations of PCR has been quantification. PCR is ideal for detection of small amounts of target signal, but without quantification, decisions on whether to treat fungal disease, or to assess the effect of the fungus are delayed until more conventional methods of quantification such as serial dilution on selective media, can be performed. Techniques have now been developed that allow quantification of PCR target signals. These include competitive PCR, which involves an additional target sequence being added to the PCR mixture. The PCR product is of a different size to the fungal DNA to be used, but is recognised by the same primers and competes for them hence the referral to competitive PCR. The added target is serial diluted across a range of PCR reactions and the level is previously quantified. Quantification is done visually on a gel, when the ratio of both target signals is the same then the level of unknown fungal DNA matches the known quantity of the added DNA and, therefore, the level of fungus in the sample can be quantified. This method has been used successfully to quantify the nematophagous fungus *P. chlamydosporia* from soil (Mauchline *et al.*, 2002).

Real-time PCR

A less time consuming process involves the use of real-time PCR, during which, the accumulation of PCR products is measured automatically during each cycle in a closed tube format using an integrated cycler/fluorimeter. Direct measurement of the accumulated PCR product allows the phases of the reaction to be monitored. The initial amount of target DNA in the reaction can be related to a cycle threshold *(ct)* defined as the cycle number at which there is a statistically significant increase in fluorescence (Figure 1).

Target DNA can then be quantified by construction of a calibration curve that relates *ct* to known amounts of template DNA. This method has been used successfully to quantify the nematophagous fungus *P. cucumerina* from field soils (Atkins *et al.*, 2003B: Figure 2).

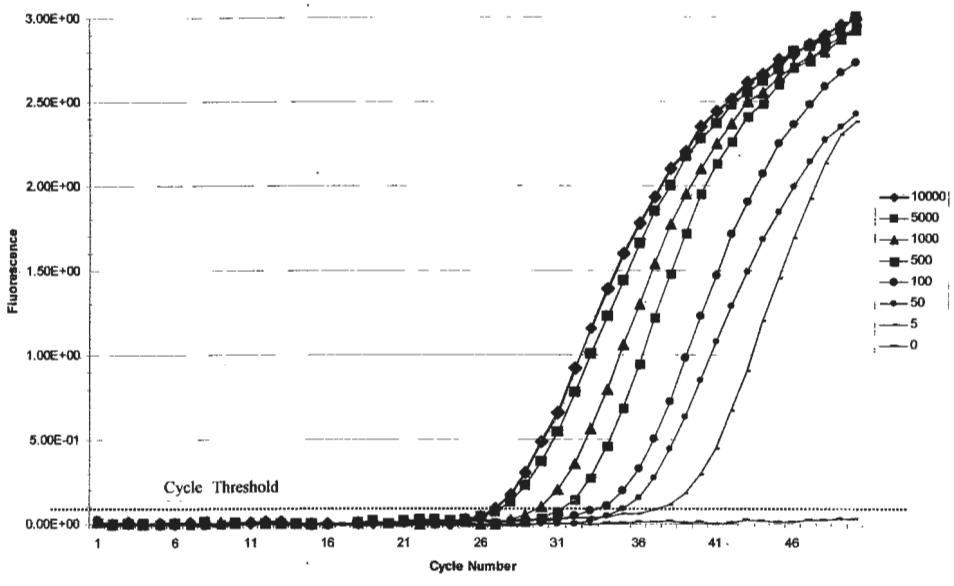

Figure 1. Real-time PCR traces of a serial dilution of *Plectosphaerella cucumerina* spores in soil. Figure shows plot of cycle threshold (ct)

PCR products can be monitored using either fluorescent DNA intercalating dyes such as SYBR Green I, or sequence specific probe based assays using TaqMan probes (Wittwer *et al.*, 1997) and molecular beacons (Tyagi *et al.*, 1998). The use of intercalating dyes is the cheapest option, but is less specific as the dye binds to all double-stranded DNA present, and primer dimers can result in a false reading. Sequence specific probes add a greater degree of specificity to the reaction (Livak *et al.*, 1995). The probe consists of a fluorescent reporter dye and a quencher, once the probe has bound and the *Taq* enzyme elongates the target gene, the reporter dye is released and fluorescence detected. The specificity of the probe allows single nucleotide polymorphisms (SNPs) to be targeted (Tyagi *et al.*, 1998), therefore, allowing isolate specific probes to be designed. The use of probes with different reporter dyes allows isolates to be monitored together, a massive step forward in trying to understand how isolates interact, and trying to understand the complexity of populations. Currently, Lux™ Fluorogenic primers are replacing TaqMan probes in real-time PCR technology to provide a cheaper, reliable method, with the specificity of TaqMan probes without some of the restraints (Nazarenko *et al.*, 2002). Real-time PCR has been used successfully to quantify a number of plant pathogens (Bohm *et al.*, 1999; Bates, *et al.*, 2001; Cullen *et al.*, 2001; Winton *et al.*, 2002) and the technique has much promise for the future.

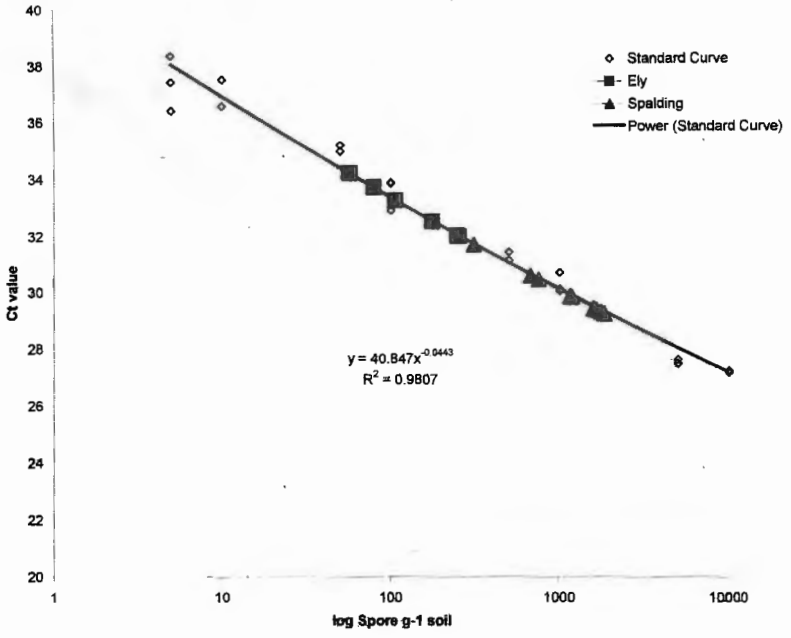

Figure 2. Standard curve of ct values of a serial dilution of *Plectosphaerella cucumerina* spores in soil. Plot shows value of *P. cucumerina* populations in two field sites: Ely, and Spalding in the UK.

Discussion

Although PCR technology can provide accurate and valuable information about a fungus, it is essential to combine this technology with other methods to provide more comprehensive data on its activity. Baiting of soil with nematode eggs (Sikora *et al* 1994; Atkins *et al.*, 2003B; Atkins *et al.*, 2003C) provides data on the ability of a fungal isolate to parasitise the nematode pest, whereas serial plate dilution provides data on the viable population of the fungus. Atkins *et al.*, (2003B) compared the use of these methods, as well as molecular techniques, to monitor the nematophagous fungus *P. cucumerina* in two field sites stressing the importance of multiple methods to investigate potential biological control agents. Table 1 provides an overview of the pros and cons of the methods reviewed in this text.

The ability to design PCR primers to target specific regions of DNA has led to a greater understanding of fungal ecology, fungal-plant interactions, fungal-pest interactions and fungal-fungal interactions. As more information becomes available on fungal genomics and gene function, the greater becomes the scope of PCR technologies.

As yet, these technologies are still laboratory based, but the aim is to develop PCR/probe based detection kits that can be taken into the field to assess: fungal diseases; the ability of a soil to be suppressive against a particular pest through naturally occurring fungal antagonists; or to monitor and assess released fungal strains, providing a new and exciting field of fungal molecular diagnostics.

Table 1. Overview of methods outlined in the paper showing brief advantages and disadvantages of each technique

Method	Advantages	Disadvantages
Selective media	Quantitative Minimum expertise necessary Measures viable propagules	Time and space consuming Quantitative? Data difficult to interpret No separation of spores and hyphae
PCR	Quick Sensitive/specific RNA techniques available to monitor viable population	Non-quantitative Expertise necessary Expensive
Real-Time PCR	Quick Sensitive/specific RNA techniques available to monitor viable population Quantitative	Expertise necessary Expensive Expensive capital equipment
Baiting	Semi-quantitative Direct monitor of BCA potential Cheap	Semi-quantitative Relies on other methods for identification

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Multitrophic Interactions in Soil and Integrated Control
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Soils suppressive to *Rhizoctonia solani* AG 2-2IIIB in sugar beet

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Abstract: Soils suppressive to *Rhizoctonia solani* AG 2-2IIIB in sugar beet have been found in the Netherlands. The occurrence of soils suppressive to *R. solani* opens the possibility for natural management of *Rhizoctonia* in sugar beet. The aim of our research is to determine mechanisms causing soil suppressiveness to *Rhizoctonia*. A bioassay was developed to screen soils for their ability to suppress *Rhizoctonia*. A strategy to analyse suppressive soils was suggested.

Key words: suppressive soil, *Rhizoctonia solani*, sugar beet

Introduction

Rhizoctonia solani (AG 2-2IIIB) causes damping-off and black root rot of sugar beet seedlings and root and crown rot of mature sugar beets. Decreases in sugar yield, tare beets and problems with storage and processing are the result of severe infestations caused by *R. solani*. *R. solani* has been known to infect sugar beets in the Netherlands since the seventies, but increased significantly as from the early nineties. The disease is most severe on light sandy soils. *Rhizoctonia* occurs at approximately 80 percent of the sandy soils in the sugar beet areas in the Eastern part of the Netherlands. In other soils of the Netherlands, the disease is less abundant. The infection of seedlings is enhanced in spring because sandy soils warm up rapidly with increasing air and soil temperatures. High temperatures (>18°C) and high humidity favour *Rhizoctonia*. Furthermore, reduced tillage, crop rotations with host plants and farmers practices causing bad soil structure may have contributed to the increase of *Rhizoctonia*.

Cultural practices that may reduce *R. solani* damage include (1) the use of *Rhizoctonia* resistant cultivars (2) providing optimal soil structure (3) crop rotations with non-host plants, (4) early sowing, and (5) control of weeds. *Rhizoctonia* resistant varieties, however, are not immune for root rot. In addition to the choice of resistant varieties, other cultural practices should be undertaken to manage the disease.

Soils suppressive to *R. solani* in sugar beet have been described in the Netherlands (Schneider, annual report of IRS 2000) and in Japan (Hyakumachi and Ui, 1982; Hyakumachi *et al.*, 1990). Suppressive soil can be defined as a soil in which disease-development is suppressed even though when the pathogen is introduced in the presence of a susceptible host (Baker and Cook, 1974). The pathogen, a susceptible host and antagonist(s), or specific abiotic factors are required to develop soil suppressiveness to *Rhizoctonia* spp. (Hyakumachi, 1996). Absence of any of these components prevents the build up of soil suppressiveness in the field. The occurrence of soils suppressive to *R. solani* opens the possibility for natural management of *Rhizoctonia* in sugar beet. The aim of our research is to determine mechanisms causing soil suppressiveness to *Rhizoctonia*.

Materials and methods

A bioassay was developed to screen soils for their ability to suppress *Rhizoctonia*. Soil samples were taken at random from different practical fields in the Netherlands in 2000 and 2001. Soil samples were not inoculated or artificially inoculated (0.5% w/w and 1% w/w) with a three-weeks-old sand/oat meal (95:5 w/w) culture of *R. solani* AG 2-2IIIB. Our standard sand : potting mixture (10:1) was used as a control. Soil was put into tubes (ø 2 cm, height of 15 cm) and sugar beets were directly sown. The bioassay comprised 24 replicates and was performed in a growth chamber at 23°C during the day, and 15°C during the night, a photoperiod of 14 h and a relative humidity of 95%. The disease incidence (percentage of diseased plants) was rated two and four weeks after sowing. The level of *Rhizoctonia*-suppression was expressed as percentage of healthy plants. Soils conducive to *Rhizoctonia* were referred to soils in which by low inoculum level (0.5% w/w) low percentages or no healthy plants were found after two weeks. Soils suppressive to *Rhizoctonia* were referred to soils in which even by high inoculum level (1% w/w) high percentages of healthy plants were found after four weeks.

Results and discussion

Soil samples taken from different practical fields in the Netherlands in 2000 and 2001 differed in the level of *Rhizoctonia*-suppression (Fig.1, 2 and 3). Soils suppressive to *Rhizoctonia* in sugar beet were found in two successive years (Fig. 2 and 3).

The occurrence of soils suppressive to *Rhizoctonia* may be the result of abiotic and biotic soil properties inducing suppressiveness (Herr and Roberts, 1980; Liu and Baker, 1980; Chet and Baker, 1981; Stephens *et al.*, 1993) or because of alterations of morphology and physiology of *R. solani* isolates (Casthanho and Buttler, 1978) as well as the reduction of virulence of *R. solani* (Castanho *et al.*, 1978). Protection of the host by induced resistance or endophytes may also contribute to the suppression of *Rhizoctonia* (Pleban *et al.*, 1995). We just started to unravel causal mechanisms of soil suppressiveness and the first experiments are being set up.

To determine mechanisms causing soil suppressiveness to *Rhizoctonia*, the strategy reviewed by Weller et al. (2002) was suggested. The strategy includes pasteurisation or irradiation of soils and use of selective biocides to determine whether soil suppressiveness can be destroyed. The strategy also includes the transfer of suppressive soil to conducive or irradiated soil to determine whether specific microbial groups are responsible for the suppression or the total microbial diversity. Microbial groups that are responsible for suppression can be characterised with molecular markers.

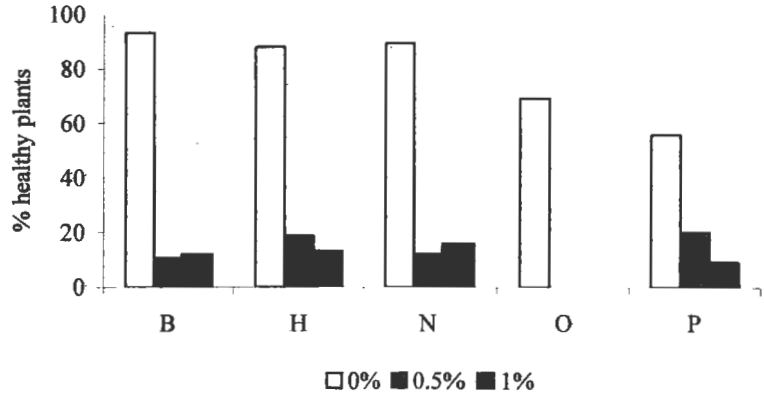

Figure 1. Level of *Rhizoctonia*-suppression (percentage of healthy plants) of soil samples at two weeks after sowing of sugar beet from different practical fields in the Netherlands in 2000. Sand : potting mixture (10:1) was used as a control. Soil samples were not inoculated and artificially inoculated (0.5% w/w and 1% w/w) with a three-weeks-old sand/out meal (95:5 w/w) culture of *Rhizoctonia solani* AG 2-2IIIB.

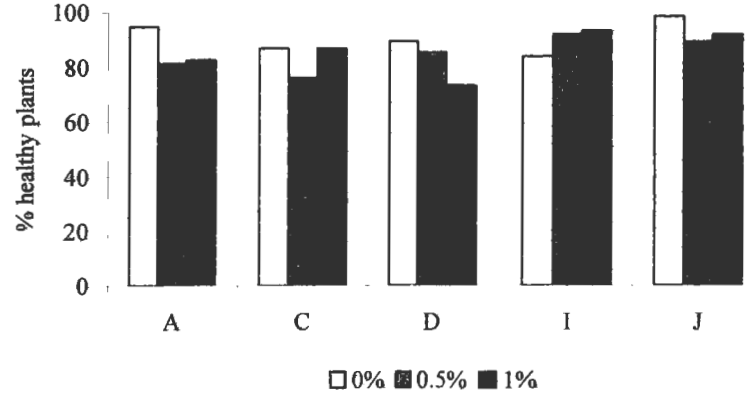

Figure 2. Level of *Rhizoctonia*-suppression (percentage of healthy plants) of soil samples at four weeks after sowing of sugar beet from different practical fields in the Netherlands in 2001. Sand : potting mixture (10:1) was used as a control. Soil samples were not inoculated and artificially inoculated (0.5% w/w and 1% w/w) with a three-weeks-old sand/oat meal (95:5 w/w) culture of *Rhizoctonia solani* AG 2-2IIIB.

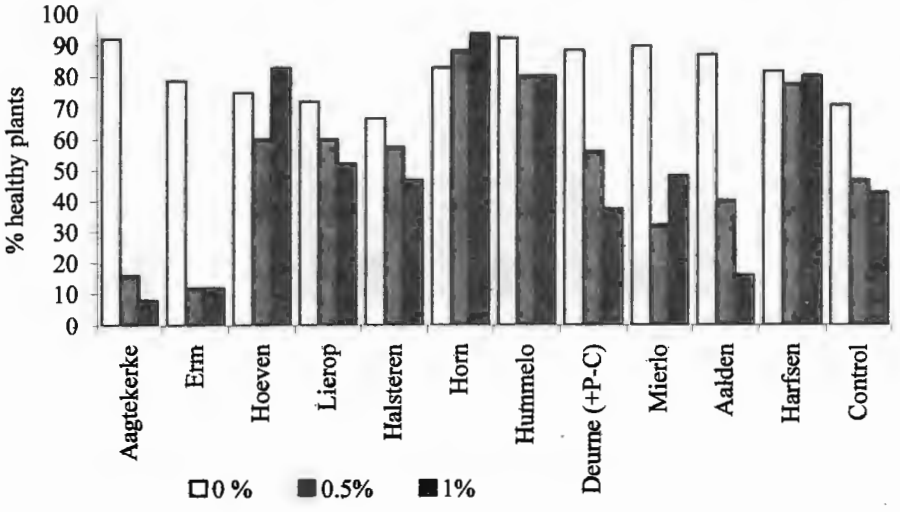

Figure 3. Level of *Rhizoctonia*-suppression (percentage of healthy plants) of soil samples at four weeks after sowing of sugar beet from different practical fields in the Netherlands in 2001. Sand : potting mixture (10:1) was used as a control. Soil samples were not inoculated and artificially inoculated (0.5% w/w and 1% w/w) with a three-weeks-old sand/oat meal (95:5 w/w) culture of *Rhizoctonia solani* AG 2-2IIIB.

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Biology and Management of a New Disease of Nursery and Small-Fruit Crops.

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Abstract: The Oomycete pathogen *Phytophthora ramorum* poses significant risks for the nursery and small fruits industry in the United States. The disease known as Sudden Oak Death (SOD) is responsible for a new epidemic of coastal forest vegetation in the west coast of the United States with an expanding host range including *Vaccinium, Rhododendron,* and several cultivated plants in the *Ericaceae* family. In Europe, the pathogen has been reported infecting both *Rhododendron* and *Viburnum.* The United States Department of Agriculture, Agricultural Research Service (USDA-ARS) is undertaking a new research program to understand the biology of this pathogen by examining soil components in disease development and rhizosphere interactions for management and control.

Key words: sudden oak death, Oomycete, soil-borne disease

Introduction

Since 1995, large numbers of *Quercus* (oak) species on the west coast of the United States have been dying in large numbers. The disease has been called sudden oak death (S.O.D.) and the cause has recently been identified as *Phytophthora ramorum.* The host range of the pathogen is unclear, but infection has been reported on cultivated species of *Vaccinium, Rhododendron,* and *Viburnum.*

For this reason, the nursery and small fruits industry in the United States is concerned. In California and Oregon, *P. ramorum* has been recovered from diseased plant material, leaf litter, and from soil which suggests that it may be transferred in soils and planting material used as components in potting media. Its biology is not completely understood, however it appears to invade hosts through wounds in stems and limbs. Phloem tissue and the outermost layers of the xylem are killed. *P. ramorum* has also been isolated from leaves, so foliar infections may serve as reservoirs for inoculum. Infection via roots is not known to occur but has not been investigated.

Soilborne pathogens such as species of *Phytophthora, Pythium, Rhizoctonia* and *Cylindrocladium* have the capacity to survive in soils for extended periods. Research on *P. ramorum* is needed to elucidate the role of soil in disease development and pathogen survival. If cultivated nursery and small fruit crops are susceptible to *P. ramorum,* there will be an immediate need to manage or prevent this disease in commercial production systems. The USDA, Agricultural Research Service intends to implement a new research project to elucidate the role of soil in disease development and possible control and management options. The research will determine if potting media and/or the components will harbor *P. ramorum* propagules, and evaluate the viability of reproductive propagules in soil components as well as growth and spread of the pathogen. Rapid field detection techniques and protocols for sampling irrigation water and soil for the presence of *P. ramorum* will be developed. ARS also seeks to determine how *P. ramorum* moves from soils in potted plants to infect and cause

disease in other plants thereby determining inoculum levels needed for infection and at what threshold. Integrated pest management and biologically-based control strategies involving beneficial microbes will be evaluated.

Material and Methods

Soil biology and host range
Various potting media and/or soil components will be evaluated to determine if they harbor *P. ramorum* propagules. Sawdust, sand and other potting soil components will be screened for the presence and types of propagules. The pathogen will be inoculated into soil components and evaluated for survival and growth. Koch's postulates will be confirmed according to the definition of the procedure, to verify host range including use of the isolate extracted from the same type of host, reproduction of symptoms, re-isolation of pathogen and confirmation that it is *P. ramorum.*

Biologically-based control options
Candidate bacterial biocontrol agents will be isolated from rhizosphere soil, potting mix components, composts and evaluated for suppression. Expressed antagonism will be carried forward to a cocktail inoculum then evaluated in seedling assays. Bacteria associated with mycorrhizal and non-mycorrhizal roots will be isolated and categorized based on antagonistic potential and capacity to influence plant growth as a result of production of phosphatases, phosphate solubilization and IAA. The efficacy of mixtures of antogonistic rhizobacteria and mycorrhizal fungi wil be evaluated by inoculation. Disease incidence and severity will be measured. Cultural practices, soil and soil-less growth media, and environmental factors will be evaluated for effects on beneficials. Strategies for incorporation and augmentation of beneficial rhizobacteria and mycorrhiza will be developed.

Chemical control options
The effectiveness of fungicide treatments on nursery stock will be evaluated. Fungistats and fungicides will be tested to determine if they mask the appearance of *P. ramorum* symptoms on nursery stock. Various fungicides will be tested for efficacy by exposing root cuttings to various chemicals.

Discussion

Soilborne microorganisms significantly influence productivity of nursery and small fruit crops. This long-term project aims to address the biology of a newly reported Oomycete pathogen with a broad host range threatening nursery and small fruits. The project will evaluate the role of beneficial microbes on disease suppression and plant growth, including mycorrhizal fungi and growth-enhancing and antagonistic rhizobacteria. Mechanisms involved in biological control etiology and epidemiology of soilborne diseases of small fruit and nursery crops will be evaluated. This research project under development will be a collaborative effort involving U.S. government agencies, university cooperators and international collaborators.

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Management of Root-knot nematodes on the agroforestry tree crop *Sesbania sesban*

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This work formed part of the project: 'The assessment of major soil borne pests of the multi-purpose agroforestry tree, *Sesbania sesban*, and the development and promotion of IPM strategies' (R6736, ZA0027) funded by the UK DFID Crop Protection Programme.

Background

The increased use of nitrogen fixing leguminous species of trees to improve soil quality is a major component of the development and promotion of agroforestry systems in Africa. One of the main agroforestry tree crops being promoted in Africa is *Sesbania sesban*, a fast growing, N_2-fixing tree with good quality foliar biomass traditionally grown in Africa and acceptable to farmers for improving soil fertility. *Sesbania* is being recommended as short-duration improved fallow crop to replenish soil nutrients and yields of sequentially grown food crops particularly maize.

The promotion of *Sesbania* as an improved fallow has been restricted partly because the tree is susceptible to soil borne pests, in particular to the root knot nematodes, *Meloidogyne* spp. Problems had arisen concerning the establishment and subsequent growth of *Sesbania* in Uganda, Rwanda, Kenya, Tanzania and Malawi.

Once plant nematodes become established in field soils it is not possible to eradicate them by any reasonable known methods including chemical pesticides (nematicides); soil populations can be reduced to low levels but they cannot be completely killed mainly because they can survive at depth in field soils. (An exception to this is long-term or regular flooding of the soils.)

The results from the project studies determined that two species of rootknot nematodes, *Meloidogyne incognita* and *M. javanica*, can be major pests of *Sesbania sesban* confirming other studies. However, there are relatively simple and practical ways of avoiding the problems caused by the nematodes.

The main reasons for severe rootknot nematode damage to *Sesbania* (and other similar crops grown with *Sesbania*) are:

1. the susceptibility of *Sesbania* to rootknot species,
2. growing *Sesbania* in soil heavily infested with the nematodes, and
3. the poor health and establishment of seedlings which are commonly infested with nematodes prior to planting in the field.

Death associated with nematode damage more often occurs in adverse growing conditions such as in sandy soils during poor rains in the initial stages of crop establishment. Most nematode damage to *Sesbania* and any plant loss normally occurs within the first year of growth with plants not surviving the first dry season. The most susceptible stage of plant

growth to nematode attack is at the seedling stage. Plants which are free of nematode infection in the early stages of growth normally are not so badly affected and are more likely to tolerate nematode damage in the later stages of growth as they mature into trees.
No true resistance was detected in provenances of Sesbania that would allow them to be used for nematode management. Therefore, the main methods to manage nematode populations and prevent excessive damage is by other means:

1. Managing Nematodes in Field Soil: reducing nematode soil populations to below a damaging level before susceptible *Sesbania* is planted, where the nematode is known to occur in the field

or

2. Managing Nematodes in Seedlings and Nursery Beds: avoiding nematode pests and preventing their introduction and spread into field soils.

Managing nematodes in field soil

Once present in soils, nematode populations have to be reduced to levels which will cause the minimum of damage to *Sesbania* when the fallow crop is planted in these soils. This is possible by rotation of non-host or resistant crops or varieties, or the use of crop-free, bare fallows.

Rotation of crops

Where nematodes are present in the soil, rotating non-host, poor host or resistant crops before susceptible crops is a means of reducing damage by the nematodes. Crops which are good hosts of the rootknot nematodes and normally are very susceptible to damage by the pests cause a build-up of soil populations. Such crops are tomato, eggplant, okra, cucurbits, tobacco, sweet potato, beans (*Phaseolus*), cowpea (*Vigna*) and other legumes. These should not be grown immediately before *Sesbania*. The crops known to be resistant or poor hosts of the rootknot nematode species are cereals, particularly maize and sorghum, and groundnuts. *Sesbania* being a susceptible host will build up soil populations to damaging levels. Fortunately, it has been shown that hybrid and, to slightly lesser extent, local maize grown in Zambia are normally non-hosts of most isolates of the nematode that have been tested. Maize is therefore not damaged by the nematode and will reduce soil populations of the pest when grown particularly for two cropping sequences. Although resistance has not been detected in *S. sesban,* it does occur in other crops that are generally susceptible, such as tomatoes and eggplant; when available, these can be used in the rotations.

Crop-free bare fallows

It is not normally acceptable to farmers to have unproductive land, but where it is possible to leave land free of crops as a bare fallow this is an effective means of reducing nematode populations in the soil because of the absence of host plants. Its effectiveness will depend on the number of weeds left in the ground which are hosts of the nematode as these will provide an inoculum of infection for the following crop.

Nematode-free field soil

As the nematodes are indigenous, no field or bush soil can be guaranteed free of nematodes. However, areas where no previous crops have been grown or where there has been a long bush fallow are most likely to contain the lowest populations of the nematodes. In these soils,

it may take a number of years of susceptible crops before the nematodes become evident as damaging pests. An area perceived to be 'good land' for a nematode susceptible crop after one year's growing may turn into a bad area for the crop the next year if the crop is grown a second or third time because of the build-up of nematodes.

Chemicals

Chemical control using nematicides is not a recommended option for farmers. Their costs, lack of availability, mammalian toxicity, difficulties of applying and general ineffectiveness make them inappropriate for use by the farmers being targeted.

Managing nematodes in seedlings and nursery beds

Avoiding nematode pests and preventing their introduction and spread into field soils.

Use of Nematode-Free Seedlings Produced from Nematode-Free Nursery Beds

When seedlings are used to propagate *Sesbania*, they can be produced free of nematodes by ensuring that nursery bed soils are free of nematodes. Nematodes are spread into nurseries in the actual nursery soil used, in other soil and plant material (eg. the sprinkling of *Sesbania* soil used to inoculate *Rhizobium*), and in some immature vegetable compost. (NB. If *Sesbania* is direct seeded they will initially be free of nematodes as rootknot nematodes are not seedborne; all infection will then be from the field soil only).

Soils for nurseries naturally free of nematodes

Dambo soils: Nematodes will not normally be found in true dambos (dimbas) which are regularly flooded each year and these areas can be considered as nematode-free soils (rootknot nematodes cannot survive in flooded, anaerobic conditions). Soil from the dambos can be dug out and moved to the nursery, or nursery beds can be actually raised in the dambos. As long as no other soil or infested material is added to the dambo soils, they will remain free of nematodes for the growing season.

Controlling nematodes in nursery bed soils

If the soil used is suspected to be infested with nematodes (any field or bush soil) it is possible to treat this relatively small amount of shallow soil by various methods.

General: It is preferable for the farmers to replace nursery bed soils each year or move beds to a new site each year to prevent nematode build-up. It is recognised that this is not always feasible or realistic with growers. However, when soil is not replaced one of the main sources of nematodes in the beds is in roots of remaining *Sesbania* seedlings left over from the previous year - these with their complete root systems should removed as early as possible. Preparation and treatment of *Sesbania* nursery beds should begin at the end of the growing season or in the dry season as long as possible before the next growing period (at least one month ie. before September/October, but longer will be more effective); preparation should not be left to the last minute just before planting new seed.

Turning nursery bed soil

New or old soil is dug and all deep rooted plants including remaining *Sesbania* seedlings from previous year are removed (easier to dig, weed out roots and break up soil into an even fine

tilth if done at the end of the growing season when soil is still moist). This finely dug soil is then left exposed and turned on a regular basis (every two weeks) during the dry season. Nematodes are killed by being exposed to higher temperatures, the sun and drying. This is possibly one of the simplest and most effective means controlling nematodes in nursery bed soils.

Burning plant debris on surface of soil
A relatively common practice with some farmers. Heat will kill the nematodes but the main problem with this method is achieving a sufficiently hot burn that causes the heat to go down into the soil and not just up into the air. This means wood rather than grass for example.

Solarization
A proven method to control nematodes if correctly applied. Clear plastic sheets are spread on the surface of moist nursery bed soils and left in place during the dry season for up to 3 months. The clear plastic has a glasshouse effect (this does not happen with black plastic) causing temperatures to increase by 5-10 C at depths of 15 - 25cm. This should be sufficient in Zambian conditions to destroy most if not all rootknot nematodes and also weeds. The economics of this method were not determined but it did show promise and could be recommended to innovative farmers who may wish to try this method although it does require more outgoings and care than other methods.

Other considerations in nursery soils
Inoculation of *Rhizobium*: the practical method recommended to farmers who wish to grow *Sesbania* is to add *Rhizobium* in the form of soil collected from around established *Sesbania* trees. As pointed out in the above sections, such soil from around trees is very likely to be infested with rootknot nematodes! To avoid infesting the carefully cleaned or selected nursery bed soil with nematodes at the last minute prior to planting seeds, it is necessary to treat the *Sesbania* soil before it is applied on the beds. Only a relatively small amount of *Sesbania* soil is involved (0.5 to 1 kg per 10 to 20m bed) and it can be treated in a similar manner to turning bed soils. If collected months before nursery preparation, the soil is spread on a hard surface (on metal or plastic sheets if at all possible) and turned regularly over the dry period. After this is done it can be safely applied to the beds.
(NB. Other methods could be used such as a form solarization in clear plastic bags turned in the sun.)

Sand added to surface of beds
This is also a standard recommendation to farmers setting up beds. Where the addition of sand is considered necessary to increase the porosity of the soil, only riverbed sand should be used as this is the least likely to be infested with nematodes.

Summary of methods for the management of nematodes on *Sesbania*

Avoiding crops in rotations which are highly susceptible to root knot nematodes and the use of nematode-free seedlings are the most effective nematode management measures that can be taken against nematode pests of *Sesbania*. Sensible manipulation of field crops using non-host crops of the nematode in sequential planting with *Sesbania* (and other susceptible crops) will limit damage by the nematodes. Most nematode damage to *Sesbania* is caused at the seedling

and early growth stage and therefore the most effective means of managing the nematodes is at the time of seedling production in nursery beds and at the establishment of the crop. Greater attention to nursery beds earlier in the season is a simple, practical and effective means of producing seedlings free of nematodes.

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Observations on the biology of *Pasteuria* parasites and microbial nematode control

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Abstract: Biological control of phytoparasitic nematodes rely on microbial soil antagonists, including specialized bacteria. Nematode populations are regulated by pathogens including *Pasteuria* forms, specific and obligate Gram+ bacteria living inside hosts. Light microscopy observations were carried out on glycerol dehydrated nematodes to identify some common biological features typical of all *Pasteuria* parasites of nematodes. Available sequences of *Pasteuria* 16S rDNA genes from different isolates were used to infer *Pasteuria* evolutionary history. The evolution and the biochemical adaptations to the parasitic behaviour were investigated and discussed. Hypothesis on *Pasteuria* evolutionary origins are proposed. The main characters promoting microbial antagonism as a suitable tool for biological control of nematodes are also discussed.

Key words: bacteria, nematodes, parasites, *Pasteuria*.

Introduction

Biological antagonists of nematodes are widespread in nature and may be isolated from different types of soil and climates. They include microparasites and predators belonging to taxa as diverse as fungi, bacteria, amebae, as well as predatory invertebrates. One of the widest group of bacerial nematode pathogens currently investigated for biological control applications is formed by *Pasteuria* spp., Gram+ endoparasites of phytoparasitic and free living species (Starr and Sayre, 1988).

The biology of parasitism shares common traits among *Pasteuria* forms and appears the result of a long-term host evolutive adaptation. These bacteria depend almost entirely for their life-cycle on the hosts' living tissues, in which vegetative branched thalli develop, following germination of an infective endospore passively intercepted by moving hosts. The parasite's sporulation phase takes place after nematode invasion and consumption, and newly formed endospores are released in soil after host cadaver destruction.

Pasteuria penetrans, initially described from root-knot nematodes *Meloidogyne* spp. as a *Bacillus* species (Mankau, 1975), was the first nematode parasite transferred to the genus *Pasteuria* (Starr and Sayre, 1988). This genus previously included only the type species, *P. ramosa*, a parasite of *Daphnia* spp. and other crustaceans. The description of *P. penetrans* and *P. thornei*, parasitic in the lesion nematode *Pratylenchus brachyurus* (Starr and Sayre, 1988) was followed by the description of *P. nishizawae*, parasitic in cyst nematodes (Sayre *et al.*, 1991). These species were later integrated by a large number of other newly discovered *Pasteuria* forms, specifically associated to distinct host nematodes (Davies *et al.*, 1990; Giblin-Davis *et al.*, 1990; Ciancio *et al.*, 1994; Sturhan *et al.*, 1994; Ciancio, 1995; Ko *et al.*, 1995).

Bishop and Ellar, 1991), and by the consequent lack of cultures deposited in microbiological collections, available for comparative studies. This problem limited the taxonomic evaluation of the genus to morphometrics and other host related observations. In the recent years, however, phylogenetic studies produced data on *Pasteuria* DNA, allowing a progressive increase of our knowledge about these microorganisms (Ebert *et al.*, 1996; Anderson *et al.*, 1999; Atibalentja *et al.*, 2000).

In this article we investigate some aspects of the biology and evolution of *Pasteuria* forms in the light of their affinity to the Bacillaceae, and discuss some practical consequences allowed by genome sequencing and analysis.

Materials and methods

Observations on *Pasteuria* parasitized nematodes were performed with light microscopy at 250-400×, on specimens extracted from soil by the sieving and decanting technique. The nematodes were examined in temporary water mounts or mounted in glycerol and stored in collection (Southey, 1970).

The evolutive history of *Pasteuria* spp. was inferred using 16S rDNA gene sequences deposited in the GenBank database, including *P. ramosa* (Table 1). On line BLAST sequence comparison (Altschul *et al.*, 1997) was performed at the NCBI public site. Sequences analysis were performed using the utilities BESTFIT, PILEUP and DIVERGE of GCG library (Wisconsin package 9.1). The DIVERGE analysis was performed on sequences aligned by PILEUP, which allowed a total of 497 codon pairs reading. The phylogenetic tree was produced by GROWTREE using the distance matrix produced by DIVERGE with the UPGMA method. The tree was then edited with Treeview (courtesy of R. D. M. Page, Institute of Biomedical and Life Sciences, University of Glasgow, UK).

A percent rate of 1.5 · 50 Myrs $^{-1}$ nucleotide synonymous substitutions per 100 synonymous sites (Ks) was used when inferring *Pasteuria* spp. divergence age. This value was obtained as average of estimated rates proceeding from fossil data and sequences of bacterial endosymbionts of insects, which showed a 1-2 % · 50 My $^{-1}$ range for 16S RNA nucleotides divergence (Ochman *et al.*, 1999).

Results

Parasitism.

Observations carried out on different *Pasteuria* forms parasitic in *Aphelenchoides* sp., *Xiphinema* spp., *Heterodera goettingiana*, *Tylenchulus semipenetrans*, *Meloidogyne* sp. and *Helicotylenchus* spp. showed a series of common events in during parasitism, which can be summarized as follows:

1. Constant associations of propagules morphotypes with hosts at the species level
2. Host invasion and destruction, lack of nematode defense
3. Absence of hypovirulence, although hosts may be partially filled by endospores
4. Resistance and persistance of endospores infectivity in dry environments
5. Infection through germination of endospores, even adhering to hosts already parasitized.

In all the different *Pasteuria* forms observed, the parasites' life-cycle always showed the following steps:

Endospores adhesion, activation and germination with loss of refractility
Host penetration through an outgrowing germ peg
Development of vegetative branched thallus and host invasion
Sporulation
Endospores release in soil

This series of events was confirmed by observations on all nematodes examined, including free-living host species, and associated *Pasteuria* forms.

Pasteuria evolution and divergence.

The nucleotide composition of *Pasteuria* 16S rDNA genes examined showed similar frequencies with <2% differences among forms parasitic in nematodes and *P. ramosa*, with exclusion of AJ243291 (Table 1). When considering nucleotide similarities, a difference greater than 5% was observed between *P. ramosa* and the nematode parasitic forms (Table 2). A remarkably lower level of nucleotide similarity was observed among sequence AJ243291 and all the other forms, which suggests that the former sequence was derived from a distinct bacterial taxon (Table 2). AJ243291 also showed a lower G+C composition (Table 1), suggesting a closer affinity to other members of Bacillaceae (probably *B. cereus*).

Table 1. Percentages of nucleotide composition in 16S rDNA genes of *Pasteuria* spp. identified by corresponding GenBank sequence accession numbers.

Accession	Species	Host genus	Nucleotide frequencies (%)				
			T	C	A	G	G+C
U34688	*P. ramosa*	*Daphnia*	18.93	23.47	24.06	33.54	57.01
AF077672	*P. penetrans*	*Meloidogyne*	18.29	24.02	22.71	34.98	59.00
AF134868	*Pasteuria* sp.	*Heterodera*	18.38	23.84	22.69	35.08	58.92
AF375881	*Pasteuria* sp.	*Belonolaimus*	18.43	23.66	22.76	35.15	58.76
AJ243291	*P. penetrans*	*Meloidogyne*	21.03	22.69	25.66	30.62	53.31

Molecular clock phylogenies may estimate the time at which two species diverged from a common ancestor, provided that data on the rate of nucleotide substitutions from different sources, including fossil records, are available. Fossil data for nematodes are scarce, and no fossil record exists for *Pasteuria* spp. Using the available divergence rate of bacteria similar to *Pasteuria* (insect endosymbionts) we attempted to infer divergence ages. The nematode parasitic *Pasteuria* spp. and *P. ramosa* divergence from a common ancestor was inferred as occurring approx. 330-357 Myrs ago (Table 3). Comparing the nematode parasitic forms, the separation of the *B. longicaudatus* form or *P. penetrans* from an ancestor in common with the *H. glycines* form was estimated as occurring approx. 95-135 Myrs ago. Finally, the lowest Ks observed between *P. penetrans* and the *B. longicaudatus* form suggests that these parasites diverged in more recent times (Tables 2, 3 and Fig. 1).

Table 2. Percentage of nucleotide similarity among *Pasteuria* spp. 16S rDNA genes, identified by the corresponding GenBank accession numbers.

	Nucleotide similarity (%)			
Accessions	U34688	AF134868	AF077672	AF375881
AF134868	92.11			
AF077672	92.78	97.35		
AF375881	92.14	96.85	99.25	
AJ243291	84.21	84.37	84.49	84.25

Table 3. Synonymous substitutions per 100 synonymous sites (Ks) and estimated age of divergence among *Pasteuria* spp. 16S rDNA genes, identified by the corresponding GenBank accession numbers (AJ243291 not included).

	Ks and divergence age (*Myrs*)		
Accessions	U34688	AF134868	AF077672
AF134868	10.64 (*354*)		
AF077672	9.90 (*330*)	4.06 (*135*)	
AF375881	10.72 (*357*)	2.86 (*95*)	0.44 (*14*)

Ebert's sequence

BLAST analysis confirmed that *Pasteuria* 16S genes include the specific oligonucleotide 5'CATCGGGAAGAAGAAATG 3'. The motif, herein named as Ebert's sequence from its first discoverer, was found in all available *Pasteuria* 16S rDNA, with the only exclusion of AJ243291, and appears as unique within Prokaryotes. The fragment occurs at positions 409 in U34688, 430 in AF077672 and AF134868, and at position 409 in AF375881. Due to its complementary ends, it forms a stemmed loop-like structure. The function of this particular region in not known, although its conservation within the genus *Pasteuria* suggests a specific metabolic role.

Germination

Observations showed that adhesion of *Pasteuria* spp. endospores always precedes their germination. Germinating endospores were also observed on nematode specimens already filled by propagules, originating from a previous infection (Table 3). Some parasitized specimens of *Helicotylenchus* sp. with germinating endospores appeared still alive but were incapable of large movements. This observation suggests that germination was not related to the efficiency of nematode metabolic activity. Endospore activation appeared related to a cuticle recognition process, after endospore attachment.

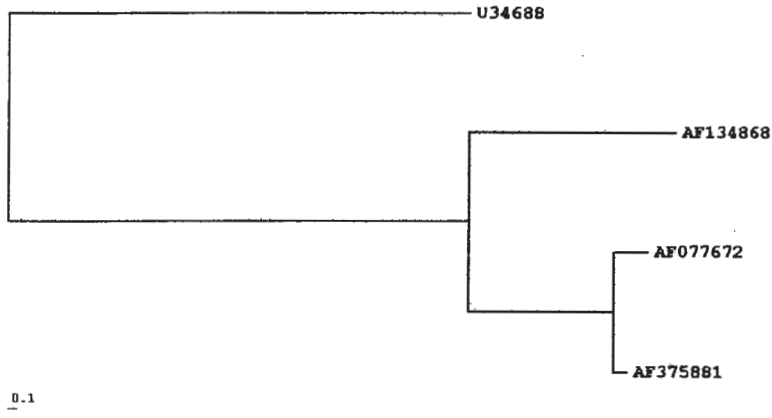

Fig. 1 Phylogenetic tree showing evolutionary divergence of *Pasteuria* spp. 16S rDNA genes (scale bar shows distances as numbers of nucleotide substitutions per site; seq. AJ243291 not included).

Table 4. Nematode species and stages already filled by *Pasteuria* propagules showing further adhering and germinating endospores.

Host	stage
Heterodera goettingiana	juvenile
Tylenchulus semipenetrans	juvenile
Helicotylenchus spp.	juvenile, adult
Aphelenchoides sp.	juvenile
Tylenchorhynchus sp.	Juv.

Discussion

Common events in *Pasteuria* parasitism suggest similar biochemical requirements conserved among isolates for activation and germination of adhering endospores. Several biological antagonists invade nematodes attaching a small propagule to their cuticle, allowing horizontal transmission of infection to new hosts through germinating spores. This strategy appeared several times during evolution of nematode microparasitism, since propagule-based infections occur among Hyphomycetes (i.e. *Hirsutella rhossiliensis, Verticillium balanoides, Nematoctonus* spp. and *Draechmeria coniospora*), as well as acquatic fungi (*Catenaria anguillulae* and *Lagenidium* spp.). The infective propagules of *Pasteuria* spp., however, are also dormant stages, capable to resist to high temperatures and dry conditions. Among nematode antagonists, this feature is typical of *Pasteuria* forms only. Dormancy and thermal resistance have useful practical implications for *Pasteuria* spp. exploitation in biological control, since they allow long-term dry storage of infective propagules (Stirling, 1991).

Previous *Pasteuria* 16S-based phylogenies showed that these microparasites represent a valid genus within the Bacillaceae, related to thermo-acidophilic bacilli of the *Alicylobacillus*

group (Ebert *et al.*, 1996; Anderson *et al.*, 1999; Atibalentja *et al.*, 2000). Inferring *Pasteuria* evolutionary origins and history may have practical consequences. The exploitation of these parasites is limited by the poor performance, survival and development shown by vegetative stages in cultures outside the nematode body (Williams *et al.*, 1989; Bishop and Ellar, 1991). Specific studies supported the coevolution of *P. ramosa* with its crustacean host (Carius *et al.*, 2001), but tests to check this hypothesis for nematode hosts and corresponding *Pasteuria* spp. remain to be developed. Data on the divergence age appear consistent with the time of nematodes appearance on earth (approx. 500 Myrs), with the only exclusion of sequence AJ243291. A strict host-parasite coevolution may suggest either the loss of specific metabolic genes by *Pasteuria*, as occurred for insect endosymbionts, and/or the evolution of specific metabolic needs for a narrow range of nutritive sources found only in the host's body. Both possibilities have important implications for *in-vitro* culture attempts.

One question concerns the possible *Pasteuria* evolution from endosymbionts or parasites of other unknown organisms. Considering that the internal living tissues of nematodes are the unique *Pasteuria* metabolic niche, some possible hypothesis may be proposed on their origins as:

1) ingested parasites or symbionts, living inside primitive microbiovorous nematodes more than 357 million years ago, developing resting forms to survive host's death or to overcome unfavourable environmental conditions;
2) or as symbionts living on cuticle exudates or metabolic by-products of primitive nematode ancestors, transmitted by contact and adhesion.
3) or as parasites originating by an host switch from parasites of other organisms.

Sequencing 16S rDNA has also some practical applications in molecular diagnosis. Ebert's sequence was found in all the *Pasteuria* 16S genes available in the GenBank database (with the only exclusion of sequence AJ243291). It appears to be a useful molecular marker for these organisms, and was exploited to detect *Pasteuria* in soil or within juveniles of *Heterodera goettingiana*. For this purpose, detection was based on PCR amplification of a 16S gene fragment containing the Ebert's sequence, followed by hybridization to highly specific DNA fluorogenic probes (Ciancio *et al.*, 2000). Due to its central position in the gene and specificity, the motif also appears helpful in PCR amplification protocols from low template amounts, when used as forward or reverse primer.

The number of bacterial antagonists of nematodes will probably increase in the future, due to the dispersion, adaptation and easy recognition of *Pasteuria* forms. Only a fraction of other nematode antagonists present in soil, however, is actually known, and it seems probable that further fastidious or unculturable bacteria will be identified in the future (Amman *et al.*, 1995).

In conclusion, the role played by bacterial communities in nematode biocontrol strategies requires further research efforts, since antagonistic bacteria hold potentials for practical purposes. Members of the *Pasteuria* group showed promising results in field trials (Nishizawa, 1987; Stirling, 1991; Gowen and Tzortzakakis, 1994; Weibelzahl-Fulton *et al.*, 1996; Chen, *et al.*, 1997). Obligate parasitic behavior holds some advantages when considering the possible exploitation of *Pasteuria* spp. as a specific product with low rizosphere impact, applied against nematode pests. Lack of mass cultivation remain a serious obstacle, expecially when considering any possible loss of key metabolic genes in *Pasteuria*. The ecology, biology and specificity of these parasites suggest, in any case, that the production of bionematicides based on durable dormant endospores will largely depend on the feasibility of low cost culture methods.

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Control of *Verticillium* wilt of cauliflower with crop residues, lignin and microbial antagonists

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Abstract: *Verticillium dahliae* var. *longisporum* is an economically important wilt pathogen in the cauliflower production. The persistent survival structures of this pathogen, the microsclerotia, are regarded as the primary target structures to control. In this study the effect of organic soil amendments (crop residues and lignin) and microbial antagonists were tested against the viability of the microsclerotia of *V. dahliae* var. *longisporum* of cauliflower. To test the effect of organic soil amendments, the wet sieving technique was used. To test the effect of microbial antagonists, a rapid screening microtiter assay was developed. The effect of organic soil amendments depended on the soil sample; but incorporating ryegrass or lignin gave the best results. The antagonists *Pseudomonas aeruginosa* PNA1, *P. fluorescens* CMR12a and *Talaromyces flavus* Vll 7b inhibited germination of microsclerotia in a microtiter assay while *Serratia plymuthica* HRO-C48 had no effect.

Key words: integrated control, green manures, biocontrol

Introduction

The soilborne pathogen *Verticillium dahliae* var. *longisporum* causes wilt in a wide range of crucifer hosts. In California, Belgium, Germany and the Netherlands, this pathogen causes severe losses in the cauliflower production. The pathogen forms highly persistent survival structures, the microsclerotia, which allow extended survival in soil. Because of their persistence in soil and their importance as infection structures, the microsclerotia are regarded as the primary target structures to control. In the past, control of *Verticillium* wilt has largely been based on chemical soil disinfestations, but because of non-selective modes of actions and environmental impact, their use is increasingly restricted by national governments. However, no economical feasible alternatives are currently available. Therefore, environmental-friendly ways to control *Verticillium* wilt on cauliflower are urgently needed. In this study we focus on using (i) organic soil amendments (broccoli residue, cauliflower residue, ryegrass, Indian mustard and lignin) and (ii) microbial antagonists (*Pseudomonas aeruginosa* PNA1, *Pseudomonas fluorescens* CMR12a, *Serratia plymuthica* HRO-C48 and *Talaromyces flavus* VII7b) to reduce the viability of the microsclerotia.

Materials and methods

Effect of organic soil amendments on the viability of the microsclerotia in naturally infested soil

Fresh broccoli residue (10% w/w), fresh cauliflower residue (10% w/w), fresh Indian mustard (5% w/w), fresh ryegrass (5% w/w) and pure lignin (0.1%, 1%, 10% w/w) were incorporated into 2 different naturally infested soil samples (Is1 and S3). Soils were incubated for two weeks and quantification of the viability of microsclerotia in soil was based on the wet sieving method described by Harris et al. (1993). The amount of crop residues that was incorporated

is more or less the same than the amount that is incorporated when using these treatments in the field (Subbarao & Hubbard, 1996; Bending & Lincoln, 1999). The lignin used throughout this study is a commercially available kraft pine lignin polymer (Indulin AT, Westvaco Co. Charleston, SC).

Effect of microbial antagonists on the viability of the microsclerotia in a microtiter assay

Pseudomonas aeruginosa PNA1 was grown in liquid glucose-casamino-acid-yeast extract (GCY) for 48h (Anjaiah et al., 1998; Tambong & Höfte, 2001), Pseudomonas fluorescens CMR12a was grown in King's B medium (KB) for 48h (Bonte, 2000), Serratia plymuthica HRO-C48 was grown in liquid synthetic medium (SM) with 0.2% (w v^{-1}) colloidal chitin for 72h (Frankowski et al., 2001) and Talaromyces flavus VII7b was grown in T2 medium for 96h (Stosz et al., 1996). To obtain the supernatans, the cultures were centrifuged for 10 min and the supernatans was collected and filter-sterilised using 0.22µm Millipore filters. Uninoculated media served as control treatments.

Dry, individual microsclerotia of Verticillium dahliae var. longisporum of cauliflower were produced as described by Hawke and Lazarovits (1994). Microsclerotia were suspended in water containing 0.08 g/l agar to obtain a homogeneous suspension of microsclerotia (Soesanto, 2000). The number of microsclerotia in the suspension was determined in small subsamples using a dissecting microscope.

Nylon mesh filters (41µm) were placed in each well of a 96 well-microtiter plate. Subsequently, 20µl of the microsclerotia solution was embedded in the filters plus 180 µl of the suspensions or the supernatants of the antagonist. After two days, the filters were retrieved and placed on a selective Soil-Pectate-Tergital-Agar (SPTA) plates (Hawke & Lazarovits, 1994). The number of germinated microsclerotia was counted after 10 days.

Results and discussion

Effect of organic soil amendments on the viability of microsclerotia in naturally infested soil

Crop residues and lignin were incorporated in two soils naturally infected with *V dahliae* var. *longisporum* microsclerotia (Figures 1a, 1b and 1c). In the Is1 soil, all the crop residues significantly reduced the amount of microsclerotia (Figure 1a). In the S3 soil, only ryegrass significantly ($p<0.05$) reduced the number of microsclerotia in soil and Indian mustard even significantly ($p<0.05$) augmented the number of microsclerotia in soil (Figure 1b). Additionally, 1% and 5% of lignin reduced the amount of microsclerotia significantly ($p<0.05$) in the S3 soil (Figure 1c). Based on these results ryegrass and lignin can be recommended.

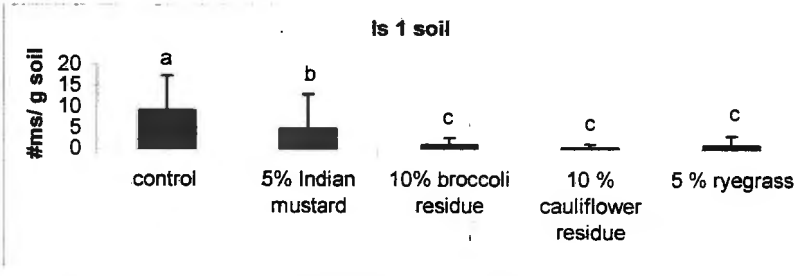

Figure 1a. Average amount of microsclerotia (ms) counted per gram of Is1 soil two weeks after incorporation of crop residues

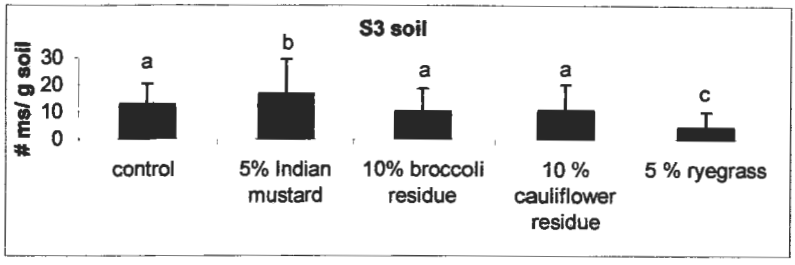

Figure 1b. Average amount of microsclerotia (ms) counted per gram of S3 soil two weeks after incorporation of the crop residues

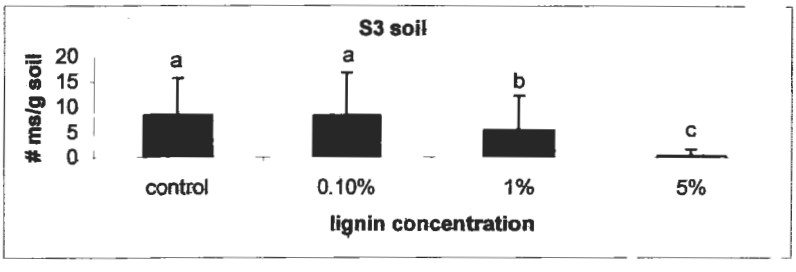

Figure 1c. Average amount of microsclerotia (ms) counted per gram of S3 soil two weeks after incorporation of lignin

Lignin is a complex molecule providing rigidity to the cell walls of plants. All crop residues contain lignin and lignin degrades slowly in soil. Only a few soil-inhabitant organisms (e.g. white rot fungi) have developed the necessary enzymes to break down lignin. Butler and Day (1999) proved that ligninases produced by white rot fungi can degrade fungal melanin. Therefore, we hypothesize that when lignin or crop residues are incorporated in soil, the lignin-degrading micro-organisms in the soil are stimulated and subsequently, also the melanin degradation of the microsclerotia is stimulated. When the melanin of the microsclerotia of *Verticillium* is degraded, the microsclerotia may become more susceptible to microbial antagonists present in the soil (Shetty et al., 2000).

Effect of microbial antagonists on the viability of microsclerotia in a mirotiter assay

Except for *Serratia plymuthica* HRO-C48, the suspensions or supernatans of all the tested antagonists, significantly ($p<0.05$) reduced the germination of the microsclerotia (Figure 2). These results indicate that *P. aeruginosa* PNA 1, *P. fluorescens* CMR 12a and *T. flavus* VII 7b are promising antagonists of *Verticillium* microsclerotia.

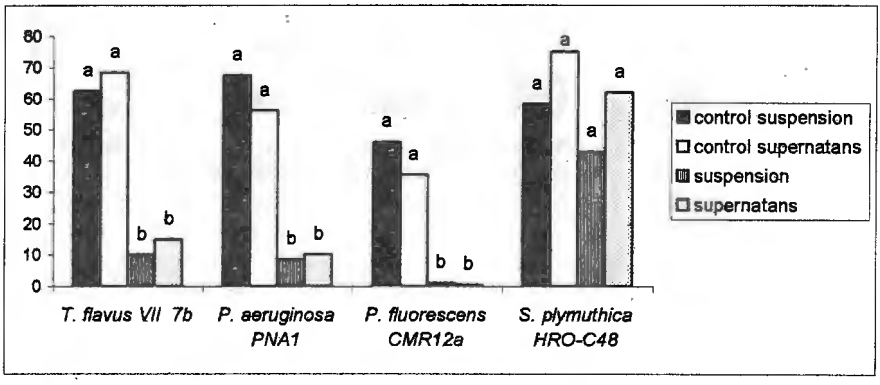

Figure 2. Average germinated microsclerotia (ms) after 2 days of incubation in a suspension, supernatans or control treatment of an antagonist in the microtiter assay

P. aeruginosa PNA1 is a phenazine-producing antagonist of soilborne pathogens such as *Pythium* and *Fusarium* (Anjaiah et al., 1998). Phenazines may also be implicated in the inhibition of microsclerotia germination. *T. flavus* VII 7b, produces glucose oxidase, that generates toxic peroxides that may affect microsclerotia (Stosz et al., 1996). The mode of action of *P. fluorescens* CMR 12a, an effective antagonist of *Pythium myriotylum* (Bonte, 2002) is unknown. *S. plymutica* HRO-C48 produces chitinases (Frankowski et al., 2001). Our results suggest that chitinases have no direct effect on microsclerotia *in vitro*.

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Challenges in the commercialisation of *Trichoderma harzianum* strain T-22, a new biocontrol agent for Europe.

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Abstract: *Trichoderma harzianum* strain T-22 has been under investigation for decades. Extensive research has shown the potential of this antagonistic fungus for the European market. At the moment T. harzianum strain T-22 is registered in the USA, Canada and Turkey (under the trade names Plantshield and Rootshield) as a biological fungicide for the control of both foliar and soil-borne diseases. Koppert is marketing this product as a plant strengthener in the UK, Norway and Iceland under the commercial name TRIANUM. In this article the hurdles that have to be taken to develop TRIANUM (and other biocontrol agents) into a commercial product for the European market are discussed.

Key words: *Trichoderma harzianum* strain T-22, registration, commercialisation, biocontrol agents.

Introduction

In the past years several authors have published about the challenges in the development and commercialisation of microbial biocontrol agents (BCA's) (Lisansky, 1997; Van der Pas et al., 2000; Jarvis, 2001; Ravensberg & Elad, 2002). Authorities all over the world are taking steps to reduce the use of and dependency on chemicals. As a result of this many broad-spectrum chemicals have been withdrawn from the market. Sometimes those chemicals have been replaced by selective, more environmental friendly chemical products. The development and commercialisation of biological, reliable, economical alternatives however still stays behind, mainly due to high registration costs and the application in niche markets.

Koppert's goal is to market effective, safe, reliable and registered bio-pesticides, that are economically interesting for both the grower and producer. In general this is a difficult task, which is expressed in the low number of available and successful microbial products on the market worldwide. Since the end of the 1990's Koppert is developing biological/natural products for the control of diseases. Due to the high costs of registration also promising products of third companies, that are (nearly) registered, are taken into investigation in order to reduce development and registration costs.

Demands of biocontrol products

In order to develop an interesting biocontrol agent demands like low production costs, long shelf-life, good efficacy, easy integration into existing IPM-systems and simple application methods have to be met. If a biocontrol agent can meet (most of) those demands it may be able to compete with the 'conventional', easy to use, effective and relatively cheap chemicals.

However even if those demands are met, it seems to be difficult to have a good turn-over of BCA products, since their use is still limited.
An overview of different commercially available microbial disease control products in the North-American and European/Mediterranean regions, based on bacteria and fungi, is given by Ravensberg & Elad (2002). In this overview *Trichoderma harzianum* strain T-22 is regarded to be the best selling bio-fungicide at the moment.

Modes of action

T. harzianum strain T-22 shows different modes of action. Most important feature of this fungal antagonist is the competition with other (pathogenic) fungi for food and place. By feeding on root exudates it removes the pathogens food supply. As *T. harzianum* strain T-22 is a strong root coloniser (forming a 'shield' around the roots of the plants) it protects the plants against fungal attack.

Promising research results

Research carried out in the USA and Europe in the past decade has showed out that *T. harzianum* strain T-22 can meet most of the demands mentioned earlier, making it a promising candidate for further development into a commercial product for the European market.
Most pronounced results obtained under European growing conditions were:

- increased yield in vegetable crops (tomato, cucumber, bean, strawberry)
- extended root development in a wide range of ornamental and vegetable crops
- enhanced growth and quality in ornamental crops (like *Kalanchoe*, *Lysianthus* and *Pelargonium*)
- disease prevention (Cosmos, cucumber, raspberry)

More information on this research is described in Dissevelt & Ravensberg (2002) and Galeano et al. (2002). Some results are presented in figure 1. In conclusion is can be stated that a healthier, stronger plant is produced by the application of *T. harzianum* strain T-22, which especially becomes visible under sub-optimal growing conditions.

Registration

At this moment T. harzianum strain T-22 is marketed in the USA, Canada and Turkey by BioWorks Inc. (as a biological fungicide (under the trade names Plantshield/Rootshield) for the control of foliar and soil-borne diseases in many different crops. Koppert has obtained the rights for marketing this antagonistic fungus in Europe and applied for registration as a plant strengthener/bio-fungicide in different European countries under the trade name TRIANUM.

At the moment there are no uniform guidelines for the registration of plant strengtheners in Europe. In Austria and Norway those products are regarded as fertilisers, for which no complicated registration procedure is requested.The UK does not request registration for plant strengtheners. Germany has developed a separate category for those products (so called 'Pflanzenstärkungsmittel'). Until recently no registration was needed in Spain, but now T. harzianum strain T-22 is on list IV of the EU-notification, registration is required, either as a pesticide or a fertiliser. In the Netherlands plant strengtheners fall under the scope of pesticides (since they influence growth processes in plants), but easier registration requirements are promised. Denmark and other European countries are awaiting the EU-guidelines to become clear.

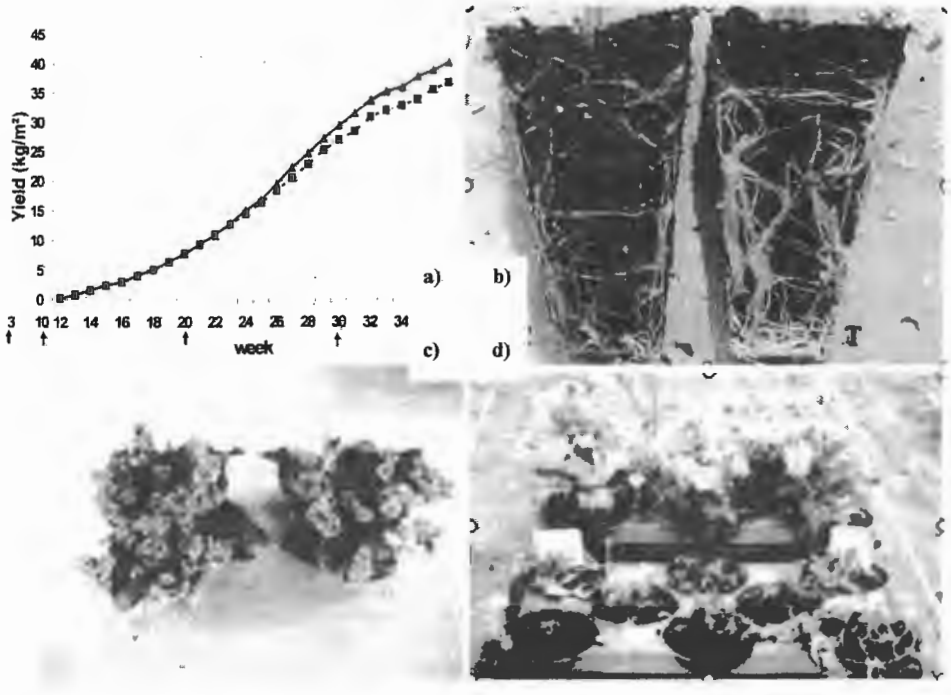

Figure 1.The effects obtained by application of *T. harzianum* strain T-22 in different crops.

a) increased yield (tomato, upper line treated plot, lower line untreated plot)
b) extended root development (sweet pepper, left untreated, right treated)
c) enhanced growth (Kalanchoe, left treated, right untreated) and
d) disease prevention (Cosmos, front untreated, background treated)

It is clear that the divers, unclear legislation and lack of harmonisation is hampering the implementation of this promising biocontrol agent. As a result TRIANUM is now only being marketed in the UK, Norway and Iceland as a plant strengthener, while registration in the Netherlands is expected next year.

Market incentives

Besides the regulatory problems also the lack of market incentives negatively influence the development and implementation of biocontrol agents. Growers are trying to differentiate their produce and to increase their market share by meeting the demands of large supermarket chains of environmentally friendly production methods. If the grower's profitability is not increased (or remains at the least stable) by increased efforts they will loose interest in the application of BCA's.

It is clear that most of the BCA's need to improve on user friendliness, efficacy, pricing and cost effectiveness in order to be interesting for both the end users and the producers. Even if BCA's can compete with chemicals on the above-mentioned characteristics, however, growers seem to be sceptical and prejudiced about the possibilities, strength and efficacy of biological products. And unless there are no (chemical) alternatives (like for example in Scandinavian countries and Canada) application of chemicals seems to be prefered. It is not clear why growers are not confident about (registered) BCA's. Perhaps their experiences with unregistered products, often with doubtful claims and results, may be one of the reasons. Not only registration, but also promotion and marketing are essential aspects in the extended use of biocontrol agents.

Experiences in the USA have shown that *T. harzianum* strain T-22 is able to compete with chemical fungicides on product costs, application costs (labour) and efficacy, thus giving growers a tool to reduce the application of and dependency on chemical fungicides.

Future outlook

In case a biocontrol agent has not been notified on the EU-list, it is forbidden since 25 July 2003. In principle the EU-guidelines will overrule over the present national guidelines. It remains to be seen what effect this enforcement will have on the availability of biocontrol agents on the European market.

At the moment the EU is in the process of making an list of products with a low risk profile exempt of registration, comparable to the GRAS (generally regarded as safe) list in the USA. Also in the Netherlands such a list exist (the so-called RUB list, on which products with low risk like milk, beer and seaweed-, garlic and onion extracts are mentioned), although the future status of this list is very unclear.

The lack of harmonisation of legislation within the EU hampers the development, commercialisation and implementation of BCA's, regardless the claims of the products (Ravensberg, 1998). Therefore it is necessary for governments to put extra effort in the harmonisation of registration guidelines and in applied research for developing good, reliable IPM-systems. In this way growers can obtain sufficient alternatives to grow their crops in an environmentally friendly, sustainable way and the industry may find a stimulus and justification for the development of safe, effective and reliable biocontrol agents.

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Antifungal activity of *Bacillus subtilis* filtrate to control *Fusarium oxysporum* f.sp. *lentis*, the causal organism of lentil vascular wilt

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Abstract: In dual cultures, the supernatant filtrate of the biocontrol agent *Bacillus subtilis* was evaluated against (*Fusarium oxysporum* f. sp. *lentis*) the causal organism of lentil vascular wilt. The antagonistic activity was evaluated as percent reduction of fungal growth (certainly due, in part, to the antifungal metabolites produced by the antagonistic bacterium). The *in-vitro* experiments showed that *B. subtilis* filtrate, whether solid or liquid media, had a strong inhibiting activity on the spore germination and mycelial growth of *F. oxysporum* f. sp. *lentis*. In a glasshouse experiment, soil was drenched with *B. subtilis* filtrate at 30 ml/kg (vol/wt) around seedlings of a susceptible lentil line (ILL 4605). In this treatment there was only 31% mortality compared with 100% kill of plants in the control treatment ($P \leq 0.05$).

Key words: *Bacillus subtilis*, *Fusarium oxysporum*, Culture filtrate, Metabolites, Antagonism, Lentil.

Introduction

Vascular wilt is one of the most important disease of the lentil (*Lens culinaris* Medikus) in Syria (Bayaa, *et el.*, 1986; Agrawal, *et al.*, 1993) and worldwide (Khare, 1981; Saxena, 1993). The causal organism, *Fusarium oxysporum* Schlecht. emend. Snyder et Hansen f.sp. *lentis*, is a soilborne fungus which can infect plants throughout the growing season (Bayaa & Erskine, unpublished). A range of metabolites with antifungal activity have been identified in antagonistic interactions involving *B. subtilis* and plant pathogens, including subtilin (Kurahshi, *et al.*, 1982), bacilysin (Vanittanakom, *et al.*, 1986), fengymycin (Loeffler *et al.*, 1986), iturins (Gueldner, *et al.*, 1988), peptidolipids (Rodgers, 1989), iturins A and surfactant (Pusey & Wilson, 1984; Hiraoka, *et al.*, 1992; Asaka & Shoda, 1996 and Ahimou, *et. al.*, 2000) and surface active properties (Thimon, *et al.*, 1992; Razafindralambo, 1998); it may produce other antibiotics as well which are inhibitory to certain root pathogens. In our previous studies, *B. subtilis* was found to give good control of lentil vascular wilt (El-Hassan, *et al.*, 1997). The objective of the present study was to produce and assess the effectiveness of antagonistic supernatant filtrates of *B. subtilis* to control or reduce lentil vascular wilt *in-vitro* and under glasshouse conditions.

Materials and Methods

Biocontrol agent, fungal material and inoculum preparation
The antagonistic bacterium *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 (IMI 388877) was isolated from Syrian lentil soils (El-Hassan, 1998) by the dilution plate technique on nutrient

agar (NA) medium. *Fusarium oxysporum* f.sp. *lentis* (isolate no. 31) was originally obtained from stems of wilted plants grown at ICARDA during the 1999/2000 crop season.

To prepare fungal inoculum, *F. oxysporum* was grown on lentil dextrose (LD) liquid medium (60g lentil seeds, 20g dextrose and 1 l distilled water) (Erskine, *et al.*, 1988) by using 150-ml of LD, in 250-ml Erlenmeyer flasks inoculated with 6 discs (5-mm dia.) obtained from margin of 2-weeks-old colony of *F. oxysporum* and incubated at 25±2 °C. After 14 days of incubation, 150-ml of sterile distilled water (SDW) was added to each flask, blended in a waering liquidizer for one minute, filtered through 6 layers of cheesecloth and microconidia density was adjusted to give a concentration of $2.5x10^6$ spore/ml^{-1} (Bayaa & Erskine, 1990; Erskine & Bayaa, 1996).

In-vitro effect of supernatant filtrate of* B. subtilis *against* F. oxysporum *f.sp. lentis

An experiment was designed to investigate the possible antagonistic effect of *B. subtilis* filtrate against *F. oxysporum* growth on solid and liquid media. The effect of the antifungal compounds of *B. subtilis* to be tested was obtained by a fermentation process using 100 ml of nutrient broth (NB), inoculated with 1 ml bacterial suspension of 24 h old *B. subtilis* culture grown on NA, incubated on a rotary shaker (150 rpm) at 25 ± 2 °C for 3 days. Bacterial cells were removed by centrifugation (Jouan centrifuge-CR3I, France) at 4100 g, 25 °C for 60 min. The supernatant filtrate was then sterilized by filtration through a sterile disposable syringe filter of 0.2 µm pores diameter (Watman, 25 mm GD/X, USA).

Solid filtrate media assay

The filtrate of *B. subtilis*, prepared as described above, supplemented with 2% dextrose and 2% agar (wt/vol) was autoclaved at 121 °C for 15 min. The *B. subtilis* filtrate plates were planted with 3-mm dia. disc from a *F. oxysporum* colony. As controls, a disc from the targeted pathogen was added to NA plates. The plates were incubated at 25±2 °C and the rate of growth inhibition was analyzed by measuring the radius growth of *F. oxysporum* colony until the control plates were fully developed. The percentage of inhibition of the pathogen growth was computed according to the following formula: $[I = 100 - (100 * R_2/R_1)]$ (Sid Ahmed, *et al.*, 1999). Where: I: inhibition of vegetative growth (mm) of *F. oxysporum*, R_1: radius of the control colony in (mm), and R_2: radius of the colony in (mm) in the tested plate.

Liquid filtrate media assay

A 50-ml sample of *B. subtilis* filtrate in 100-ml Erlenmeyer flasks, supplemented with 2% dextrose (wt/vol), was then tested for its antagonistic effect on mycelial growth and conidial germination using the method developed by Edwards (1993) and described by Walker *et al.* (1996). The flasks were then inoculated with 2 discs (3-mm dia.) taken by two-weeks-old colony of *F. oxysporum* and incubated on a rotary shaker (150 rpm, 25 ± 2 °C, 10 days). The test medium was replaced with the same volume of sterile medium of NB for the control. After incubation, the growth of *F. oxysporum* was assessed by harvesting the mycelium by centrifugation process. The samples were dried in the oven at 45 °C for 4 hours. At the same time, 1-ml aliquots of supernatant filtrate containing *F. oxysporum* were seeded in 9 ml of sterile NB, the inhibition of spore germination of the pathogen were determined microscopically after 24 h by estimating the number of germinated spores from the total using a haemocytometer (Fuchs chamber, 0.2 mm depth) at x200 magnification. Data were analyzed by GenStat Fifth Edition and means were compared by using a least significant difference (LSD) test at $P \leq 0.05$.

In-vivo effect of supernatant filtrate of* B. subtilis *against* F. oxysporum *f.sp. lentis
The experiment was conducted in trays each containing 2 kg soil under glasshouse conditions at 25±5 °C. Loamy soil and silver sand were autoclaved separately for 30 min at 121 °C three times and then mixed at 2:1 (v/v) ratio. Lentil seeds (ILL 4605) were surface-sterilized with 5% bleach for 5 min, rinsed three times in sterile distilled water and then dried in flowing sterile air for 2 to 3 h. Ten seeds were sown in each tray. Two weeks after planting, each tray was inoculated with 60 ml suspension of $2.5x10^6$ spore/ml^{-1} of *F. oxysporum* (Erskine & Bayaa, 1996). At the same time, 60-ml sample of *B. subtilis* filtrate (30 ml/kg soil), prepared as described above, was dripped around the seedlings. Wilt incidence was recorded (mostly 48 days after sowing) as the percentage of wilted plants in each tray (Bayaa & Erskine, 1990). The experiment was set-up in a randomized block design with 3 replications.

***Statistical analysis*:**
The percentage values of wilt incidence were transformed to their square root values before conducting analysis of variance. All data were analyzed according to standard analysis of variance (ANOVA) procedures by GenStat Fifth Edition. Significant differences among means were determined with least significant difference (LSD) test at $P \leq 0.05$.

Results

In-vitro effect of supernatant filtrate of* B. subtilis *against* F. oxysporum *f.sp. lentis
Bacillus subtilis is not a typical rhizobacterium; it also occurs in the soil surrounding the root (Miller, *et al.*, 1989). However, it has often been reported as an antifungal agent against plant pathogens (Huber, *et al.*, 1987; Fravel, 1988; Hebbar, *et al.*, 1991; Milus & Rothrock, 1993). In this study the supernatant filtrate of *B. subtilis* significantly reduced fungal growth as well as percent spore germination of *F. oxysporum* on solid and liquid filtrate medium compared with control treatment (Table 1).

Table 1: Effect of *B. subtilis* filtrate against *F. oxysporum* f.sp. *lentis* on solid & liquid media.

Treatment	% Inhibition of fungus growth on solid filtrate (mm)	Dry weight of fungus growth on liquid filtrate (g)	No. of spores germinated $\log_{10}(x+0.5)$ cfu/ml^{-1}	
			Germinated	Total
Control	0.00*	0.882	3.544	4.699
B. subtilis filtrate	100.00	0.00	0.301	0.301
LSD ($P \leq 0.05$)	2.618	0.005	0.029	0.013
SEM	0.667	0.002	0.007	0.005

(*) Each value is an average of results from three figures. SEM: Standard error of means.

In-vivo effect of supernatant filtrate of* B. subtilis *against* F. oxysporum *f.sp. lentis
Results of glasshouse experiment revealed that *B. subtilis* filtrate reduced incidence of vascular wilt caused by *F. oxysporum* f.sp. *lentis* on lentil plants. The antifungal activity of *B.*

subtilis might be attributed to the formation of antifungal metabolites, which have strong activity against the pathogen. At each sampling date, wilt incidence was less than the inoculated control. In soil drenched with *B. subtilis* filtrate (30 ml/kg soil) there was a significant decrease in wilt incidence and only 31% (SQRT 31=3.93) of plants died compared to 100% plants killed (in the final score) in the corresponding controls during 73 days growth (Table 2).

Table 2: Effect of the treatment with *B. subtilis* filtrate on the infection of lentil plants by *F. oxysporum* f.sp. *lentis* at time interval in sterilized soil under glasshouse.

Treatment	% Wilt incidence over time (days) SQRT(%+0.05)					
	48	53	58	63	68	73
Untreated	0.00*	0.00	0.00	0.00	0.00	0.00
Fusarium alone	5.48	7.05	8.95	9.49	10.00	10.00
B. subtilis filtrate + *Fusarium oxysporum*	0.32	1.20	2.62	3.94	3.94	3.94
LSD ($P \leq 0.05$)	0.673	1.551	1.454	0.812	0.770	0.770
SEM	0.225	0.517	0.485	0.271	0.257	0.568

(*) Each value is an average of results from three figures. SEM: Standard error of means.

Discussion

The suppression of *B. subtilis* filtrate towards soilborne fungal plant disease has been described in only a few papers (Wright & Thompson, 1985; Huber, *et al.*, 1987; Fiddaman & Rossall, 1993). Similar results were obtained by other using *B. subtilis* filtrate (Fravel, 1988; Hebbar, *et al.*, 1991; Milus & Rothrock, 1993). However, the results obtained from this study confirmed that *B. subtilis* filtrate is effective to control *F. oxysporum* f.sp. *lentis* in lentil under certain conditions. It indicates that *B. subtilis* has produced an antifungal compound or compounds, which have strong activity against the pathogen and reduces wilt incidence of lentil. Further studies are needed to identify the characteristics of the antibiotic produced by *B. subtilis* strain to enhance the potentiality of *B. subtilis* as a biocontrol agent which controls *F. oxysporum* f.sp. *lentis*.

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Evaluation of *Trichoderma hamatum* for antagonistic activity against lentil vascular wilt, *Fusarium oxysporum* f. sp. *lentis*

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Abstract: In dual cultures, the biocontrol fungus *Trichoderma hamatum* was evaluated for antagonistic ability against *Fusarium oxysporum* f.sp. *lentis* the causal organism of lentil vascular wilt. The efficacy of *T. hamatum* was evaluated as a percent reduction of fungal growth and spore production. The *in-vitro* experiments showed that *T. hamatum* had a strong activity on the mycelial growth and spore production of *F. oxysporum* f.sp. *lentis*. *In-vivo*, soil treatments with different concentrations (10^6, 10^8 and 10^{10} cfu/g^{-1} soil) of *T. hamatum* increased the population of the biocontrol fungus and reduced the population of the pathogen in two types of soil. *T. hamatum* reached a population density greater than 10^9 cfu/g^{-1} in soil 2 weeks after planting a susceptible lentil line (ILL 4605) either alone or in the presence of the pathogen.

Key words: *Trichoderma hamatum*, *Fusarium oxysporum*, antagonistic activity, lentil.

Introduction

Vascular wilt, caused by *Fusarium oxysporum* f.sp. *lentis*, is one of the most important diseases of the lentil (*Lens culinaris* Medikus) in Syria (Bayaa *et el.*, 1986; Agrawal *et al.*, 1993) and worldwide (Khare, 1981; Saxena, 1993). Microbial antagonism is an important component in biological control of soilborne plant pathogens (Baker & Cook, 1974). The potential of different species of *Trichoderma* as a biological control agents to suppress disease caused by *Fusarium* spp. has been evaluated on a number of crops (Sivan & Chet, 1986). The suppression of soilborne fungal plant disease by *T. hamatum* has been described in only a few papers (Harman *et al.*, 1980; Wright & Thompson, 1985; Huber *et al.*, 1987; Nelson *et al.*, 1988; Fiddaman & Rossall, 1993).

The objectives of this paper were to evaluate the effectiveness of the antagonistic fungus *T. hamatum* in controlling or decreasing *F. oxysporum* f. sp. *lentis* in dual culture plates, and to study the relation between rhizosphere populations of *T. hamatum* at different concentrations on growth of *F. oxysporum* in two types of soil.

Materials and Methods

Fungal cultures and inoculum preparation

Fusarium oxysporum f. sp. *lentis* (isolate no. 31) was originally obtained from stems of wilted lentil plants. *F. oxysporum* f. sp. *lentis* inoculum was prepared as described by Erskine *et al.* (1990). *Trichoderma hamatum* (IMI 388876) was isolated from Syrian lentil soils by the plate-dilution technique on potato dextrose agar (PDA) medium (El-Hassan, 1998). *Trichoderma* inoculum was mass-produced on peat-based compost plus 10% coarse grain seeds of lentil and 2% glucose for soil treatment.

***In-vitro efficacy of* T. hamatum *against* F. oxysporum**

Dual culture plates were used to evaluate the possible antagonistic effects of *T. hamatum* against *F. oxysporum* growth on PDA medium. The PDA plates were inoculated with 2 discs (3 mm dia.) collected from the growing margins of fresh *T. hamatum* and *F. oxysporum* cultures. The plates were incubated at 25±2°C for 10 days and the rate of growth inhibition was taken by measuring the radial growth of the *F. oxysporum* colony until the control plates were fully developed. The percentage of inhibition of the pathogen growth was estimated daily up to 5 days according to the following formula: $[I = 100 - (100 * R_2/R_1)]$ (Sid Ahmed *et al.*, 1999), where: I: inhibition of vegetative growth (mm) of *F. oxysporum*, R_1: radius of the control colony in mm, and R_2: radius of the colony in mm in the tested plate.

At the same time, 2 discs containing *F. oxysporum* were placed in 9 ml of sterile distilled water plus Tween 20, the numbers of spore were determined microscopically using a haemocytometer (Fuchs chamber, 0.2 mm depth) at 200-fold magnification.

***In-vivo survival of* T. hamatum *and* F. oxysporum**

The laboratory model experiment was conducted in plastic Petri plates (15 cm dia.) each containing sterilized sandy-loam and -peat soil (1:2 vol/vol) under laboratory conditions at 25±5°C. The initial cfu of *Trichoderma hamatum* per g soil was estimated before sowing to give final concentrations of 10^6, 10^8 and 10^{10} cfu g^{-1} soil using TSM. Two surface-sterilized lentil seeds (ILL 4605) were sown in each plate. Two weeks after planting, each plate was inoculated with 10 ml suspension of $2.5x10^6$ spore ml^{-1} of *F. oxysporum* (Erskine & Bayaa, 1996). The soil subsamples were drawn on 0 day and thereafter at 14-day intervals up to 56 days. Samples were serially diluted and 0.2 ml aliquots plated on TSM and the population densities of *T. hamatum* was expressed as cfu g^{-1} soil. The experiment was set-up in a randomized block design with 4 replications.

Data anlysis:

All CFU data were subjected to log10 transformation and analyzed according to standard analysis of variance procedures by GenStat Sixth Edition. Significant differences among means were determined with least significant difference (LSD) test at $P \leq 0.05$.

Results

***In-vitro efficay of* T. hamatum *against* F. oxysporum**

The percent of growth inhibition was particularly well developed with the increase in the age of the fungal culture (Figure, 1, a). In this study *T. hamatum* significantly reduced fungal growth (61.43% in day five) as well as percent spore production of *F. oxysporum* on PDA medium compared with control treatment (Figure 1, a & b).

***In-vivo efficay of* T. hamatum *against* F. oxysporum**

The soil-plate experiment revealed that *T. hamatum* proliferated and survived well in the two types of soil with most of the concentrations applied (10^8 and 10^{10} cfu/g soil), either alone or combined with the fungal pathogen *F. oxysporum* f.sp. *lentis*. It is likely that the high levels of *Trichoderma* concentrations (10^8 and 10^{10} cfu/g soil) used in this experiment overwhelmed and out-competed the plant pathogenic fungus. At each sampling date, the lowest concentration (10^6 cfu/g soil) growth only slightly exceeded the initial application rate and the highest concentration densities, as determined by TSM, remained at high rate throughout the experiment (Figure 2). In soil treated with different concentrations of *T. hamatum* there was a significant increase in population of *T. hamatum* either alone or with the pathogen (Figure 2).

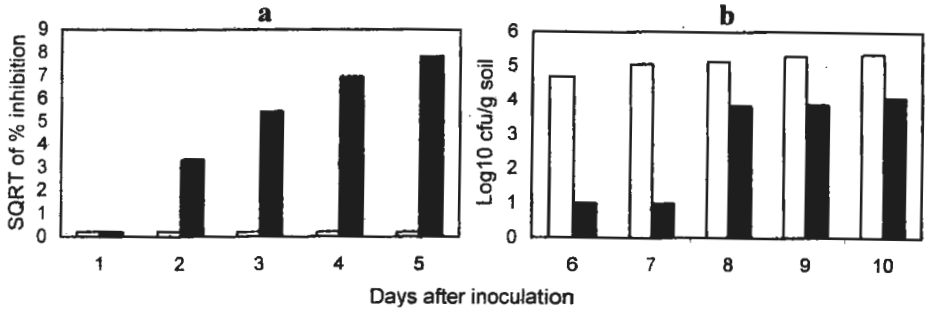

Figure 1. Growth inhibition and spore production of *F. oxysporum* by *T. hamatum* on PDA plates at 25±2 °C (□: *F. oxysporum* alone; ■: *F. oxysporum* + *T. hamatum*). **a**, growth inhibition (%) of *F. oxysporum* hyphae. **b**, spore production (cfu) of *F. oxysporum*.

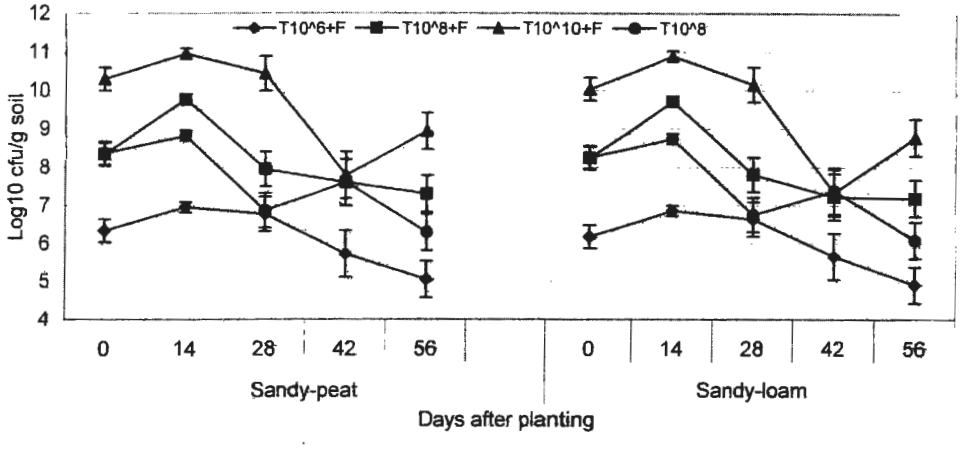

Figure 2. Effect of the soil treatment with different concentrations of *T. hamatum* on the growth of *F. oxysporum* in sterilized sandy-peat and –loam soil over 56 days. Error bars represent 5x standard errors of differences of means (Data are means of 4 replications).

Discussion

The results of these *in-vitro* and *in-vivo* studies evaluating the effectiveness of biological control fungus, *T. hamatum*, have demonstrated successful control of *F. oxysporum* f.sp. *lentis* and these results suggest that it acts through a competition for nutrient and space. The *in-vitro* results indicate that *T. hamatum* was not only able to inhibit the mycelium growth of the pathogen but also reduced the production of spores. The *in-vivo* results revealed that *T. hamatum* proliferated well in two types of soil and the population had reached its maximum level up to 15 days and thereafter the increase was less. Different initial concentrations of *T.*

hamatum had no influence on cfu in both types of soil at all the sampling dates, suggesting that the soil has slight effect on the growth of *T. hamatum* in soil. However, the results obtained from this study confirmed that *T. hamatum* is effective in controlling *F. oxysporum* f.sp. *lentis* under certain conditions.

Acknowledgements

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Multitrophic Interactions in Soil and Integrated Control
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Biosurfactants and biological control of plant pathogens

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Abstract: Biosurfactants are produced by a variety of microorganisms and have been shown tobe involved in bioremediation of xenobiotics and biological control of plant pathogens. Several strains of soil and rhizosphere-inhabiting *Pseudomonas* species were shown to produce biosurfactants and have received increasing attention during recent years as potential biocontrol agents of plant pathogenic oomycetes on different crops. Low- and high-molecular weight biosurfactants are of amphiphilic nature, with a polar part consisting of saccharides, carboxylic acids, peptides or polar amino acids and a non-polar part consisting of fatty acids or apolar amino acids. Information on the genetic basis for regulation, synthesis, and transport of biosurfactants is still sparse. Availability of biosurfactant mutants allows us now to investigate the number and identitiy of genes involved in biosurfactant production and hence to better understand and optimize biosurfactant-mediated biological control of plant pathogens.

Keywords: *Pseudomonas*, *Phytophthora*, biosurfactant, , rhizosphere,

Introduction

Biosurfactants or bioemulsifiers are microbial compounds that alter the conditions prevailing at a surface or interface (Cooper *et al.*, 1980, Fiechter, 1992, Nielsen *et al.*, 2002, Ron *et al.*, 2001, Zajic *et al.*, 1984). Several different genera in the fungal and bacterial kingdom, including *Candida, Torulopsis, Pseudomonas,* and *Bacillus* spp., are known to produce biosurfactants (Georgiou *et al.*, 1992, Ron *et al.*, 2001, Stanghellini *et al.*, 1997). *Pseudomonas* species are common inhabitants of soil and rhizsosphere environments and continue to be the focus of attention in the areas of bioremediation of xenobiotics and biological control of plant pathogenic fungi and oomycetes.

Surfactant-producing *Pseudomonas* spp. have been shown to facilitate degradation of ubiquitous pollutants such as polycyclic aromatic hydrocarbons (PAHs) and n-alkanes (Arino *et al.*, 1996, Déziel *et al.*, 1996). Recently, the potential of biosurfactants produced by *Pseudomonas* spp. in controlling plant pathogenic fungi and oomycetes was recognized. In the following sections we will focus on current progress on the use of biosurfactant-producing *Pseudomonas* strains to control plant pathogens.

Results and discussion

Biological properties of biosurfactants

Rhamnolipids produced by strains of *Pseudomonas aeruginosa* were shown to be highly effective against plant pathogens, including *Pythium aphanidermatum, Plasmopara lactucae-radicis* and *Phytophthora capsici* (Stanghellini *et al.*, 1997). Purified rhamnolipids caused cessation of motility and lysis of entire zoospore populations within less than 1 min.

Introduction of a rhamnolipid-producing strain in a recirculating hydroponic system gave good control, although transient, of *Phytophthora capsici* on pepper (Stanghellini *et al.*, 1997). Kim *et al.* (2000) confirmed and extended these observations by showing that rhamnolipid B produced by *Pseudomonas aeruginosa* B5 not only has lytic effects on zoospores, but also inhibitory activity against spore germination and hyphal growth of several other pathogens. Mycelial growth of *Phytophthora capsici* and spore germination of *Colletotrichum orbiculare* were inhibited *in vitro* and the diseases caused by these pathogens were suppressed in pepper and cucumber plants, respectively, by application of purified rhamnolipid B to leaves (Kim *et al.*, 2000).

Several cyclic lipopeptide surfactants with antibiotic properties were recently proposed as biological compounds for the control of plant pathogenic fungi (Miller *et al.*, 1998, Nielsen *et al.*, 1999,). Viscosinamide, a lipopeptide produced by soil-inhabiting *Pseudomonas* strain DR54, was shown to induce encystment of *Pythium* zoospores, and adversely affect mycelium of *Rhizoctonia solani* and *Pythium ultimum*, causing reduced growth and intracellular activity, hyphal swellings, increased branching and rosette formation (Hansen *et al.*, 2000, Thrane *et al.*, 1999, 2000). The specific cyclic lipopeptide amphisin produced by *Pseudomonas* spp. DSS73 appeared to play an important role in surface motility of the producing strain, allowing efficient containment of root infecting plant pathogenic fungi (Andersen *et al.*, 2003). Furthermore, in combination with cell wall-degrading enzymes of *Trichoderma atroviridae*, lipodepsipeptides produced by the pathogen *Pseudomonas syringae* pv. *syringae* acted synergistically in antagonism toward various plant pathogenic fungi (Fogliano *et al.*, 2002). Collectively, these studies clearly indicate the potential of biosurfactant-producing bacteria for crop protection.

Recently, we isolated 6 biosurfactant-producing *Pseudomonas* isolates with zoosporicidal activities from the rhizosphere of wheat. Application of *Pseudomonas* isolate SS101 to soil or rockwool provided effective control of several oomycetes, including *Pythium intermedium* and *Pythium aphanidermatum* on hyacinth bulbs and cucumber seedlings, respectively.

Chemical properties of biosurfactants

Biosurfactants are categorized into high- and low-molecular mass compounds. The low-molecular biosurfactants include glycolipids and lipopeptides, such as rhamnolipids and surfactin. The high-molecular compounds include proteins, lipoproteins or complex mixtures of these polymers. Although biosurfactants are structurally diverse, they all have an amphiphilic nature, i.e. they contain both hydrophobic and hydrophilic groups. The hydrophobic moieties are usually saturated, unsaturated or hydroxylated fatty acids, or apolar amino acids like leucin or isoleucin. The hydrophilic moieties consist of mono-, di-, or polysaccharides, carboxylic acids, polar amino acids or peptides (Ron *et al.*, 2001). The compounds produced by *Pseudomonas* isolate SS101 in our lab were extracted and purified by reverse phase high-performance liquid chromatography. Several fractions tested positive for biosurfactant activity. Identification of these fractions by liquid chromatography-mass spectrometry and nuclear magnetic resonance is ongoing.

Molecular characterization of biosurfactant production

Genes involved in synthesis, regulation and transport of biosurfactants produced by *Pseudomonas* isolate SS101 are currently being investigated. Tn*5* and plasposon mutagenesis generated 5 and 24 biosurfactant-deficient mutants, respectively. Mutants were characterized phenotypically by testing for foam formation, drop collapse, and their ability to reduce surface tension of water. Activity of mutants against zoospores of oomycete pathogens was compared to that of their parental wild type strain. Preliminary analysis showed that none of the tested

biosurfactant-deficient mutants reduced disease to the same extent as the wild type isolate SS101 in cucumber bioassays. Sequencing and characterization of the flanking regions of the Tn5 or plasposon inserts are ongoing.

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Plant tolerance for managing plant parasitic nematodes

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Abstract:The crops potential to tolerate damage caused by plant parasitic nematodes and still produce high yields is an important economic feature and can be used as cultural practice within integrated pest management. This is especially the case when no nematode control mechanisms are available. However, its use is still restricted to few cropping systems due to our limited understanding regarding occurrence and functioning of tolerance. In field experiments a broad spectrum of sugar beet cultivars and hybrids was tested for its tolerance towards the beet cyst nematode *Heterodera schachtii*. Three cultivars/hybrids were then selected for further field and greenhouse studies on the mechanisms of tolerance: 'Penta' (intolerant-susceptible), Stru1915 (tolerant-susceptible) and 'Nematop' (tolerant-resistant). In field experiments the degree of tolerance varied between years and was most pronounced under dry summer conditions, mainly due to the fact that tolerant plants suffered less from water stress. In greenhouse experiments, tolerant plants showed higher compensatory growth and rooting depth. Especially root length and shoot fresh weight were positively correlated with tolerance and therefore should be considered as early indicators for tolerance.

Key words: tolerance, sugar beet, *Heterodera schachtii*

Introduction

Crop tolerance towards plant parasitic nematodes is an important economic feature due to its potential to produce yield even under severe pest or disease infestation. For sedentary endoparasitic nematodes plant damage mainly occurs within the initial phase of nematode establishment through wounds caused by nematode migration within the root tissue. Such initial damage is especially critical for crops that develop storage organs derived from the root tissue such as beets. For example, nematode penetration of sugar beet tap roots can cause growth inhibition and deformation of the beet tuber. Tolerant plants can overcome this initial nematode damage and support normal root development (Evans and Haydock, 1990; Heijbroek, 1977). Therefore, in tolerant plants yield remains high even under severe nematode infestation while in intolerant plants it decreases with increasing nematode densities (Lauenstein, 1997; Mulder, 1994; Trudgill, 1991).

Regarding its enormous contribution to yield security, plant tolerance is still under-represented in research and breeding programs. More knowledge on the existence of tolerance in plants to nematodes and the mechanisms behind it would enable a broader use of tolerance not only in breeding programs but also within integrated pest management.

The objectives of the following studies were: (1) to identify sugar beet cultivars with tolerance towards the beet cyst nematode *Heterodera schachtii*, (2) describe the effect of tolerance on crop yield and (3) study the suitability of root growth parameters as an early indicator for tolerance. The potential use of tolerance for managing plant parasitic nematodes is also discussed.

Material and Methods

Field studies

The yield response of tolerant and intolerant cultivars and hybrids was studied on the experimental field of the Biologische Bundesanstalt für Land- und Forstwirtschaft in Elsdorf, Germany over the years 1999 and 2000. The experiment was established within the rotation winter wheat - winter barley - sugar beet. The following sugar beet cultivars and hybrids were tested: 'Penta' (intolerant-susceptible, I-S), 'Patricia' (partially tolerant-susceptible, PT-S), 'Tatjana' (partially tolerant-susceptible, PT-S), Stru1915 (tolerant-partially resistant, T-PR) and 'Nematop' (tolerant-resistant, T-R). In 2000 'Patricia' was replaced by 'Paulina' (tolerant-resistant). In 1999, the experiment was run at low and high population densities of *H. schachtii*, that was achieved by growing susceptible or resistant oil radish and oil-seed rape as green manure crops after winter barley and before sugar beet. Initial population densities in spring 1999 averaged 540 and 3389 eggs and juveniles (E+J) per 100 ml soil at the low and high level, respectively. In the year 2000 the initial nematode levels varied between 43 and 5930 E+J per 100 ml soil. A grouping in low and high nematode levels was not possible. Therefore, each cultivar/hybrid was tested over the spectrum of infestation levels. The sugar beets were grown in three blocks, each block comprising 12 rows of sugar beet, i. e. one border row on each side with cultivar 'Penta' and two rows each of the five cultivars/hybrids. Each cultivar within a block was subdivided into 12 replicates with each plot measuring 4.5 x 0.9 m. In 1999 the sugar beets were planted on April 1st and harvested on October 5-7th and in 2000 planted on April 11th and harvested on October 16-18th. The average number of beets per plot was 38.

Greenhouse studies

Greenhouse studies were conducted to test the hypothesis if rooting depth in the presence of *H. schachtii* is less effected in tolerant plants compared to intolerant plants and/or if compensatory growth occurs in tolerant plants. For both studies the intolerant-susceptible cultivar 'Penta' and the tolerant-resistant cultivar 'Nematop' were selected. To measure rooting depth, the sugar beets were grown in plastic tubes of 50 cm height and 5 cm diameter filled with sand. After three weeks, half of the tubes of each cultivar were inoculated with 2000 second-stage juveniles of *H. schachtii* while control plants remained non-inoculated. The experiment was terminated ten weeks after nematode inoculation. Total fresh shoot weight was recorded. The tubes were then cut lengthwise in five cores of 10 cm length and each core was separately analysed for root length. Root length was determined by scanning the root system in water on a standard scanner using the software WinRhizo version 2001a (Regent Instruments Inc., Quebec, Kanada). Each treatment was replicated eight times. To study compensatory growth, 20 sugar beets per treatment were germinated in seedling trays filled with sand. After 14 days the seedlings were carefully uprooted and from ten plants each, 50 % of the tap root was mechanically removed using a razor blade. All plants were than replanted into 300 ml plastic pots filled with autoclaved sand. The experiment was terminated six weeks after transplanting when shoot fresh weight and root length was determined.

Results

Field studies

In 1999, total yield at low nematode densities varied between 71.8 t/ha for 'Nematop' (T-R) and 80.0 t/ha for Stru1915 (T-PR) and at high nematode levels between 63.9 t/ha for 'Penta' (I-S) and 75.8 t/ha for Stru1915 (T-PR) (data not shown). Differences in tolerance between

the five cultivars/hybrids became evident when the yield of the sugar beets at low and high nematode densities was compared. Differences in sugar beet yield ranged from – 10.2 t/ha for the intolerant-susceptible cultivar 'Penta' up to + 1.6 t/ha for the tolerant-resistant cultivar 'Nematop' (Fig. 1). The other cultivars and hybrids were intermediate showing some tolerance towards *H. schachtii*. In 2000 total sugar beet yield tended to be slightly lower than in 1999, but the differences in tolerance between the five cultivars/hybrids were similar.

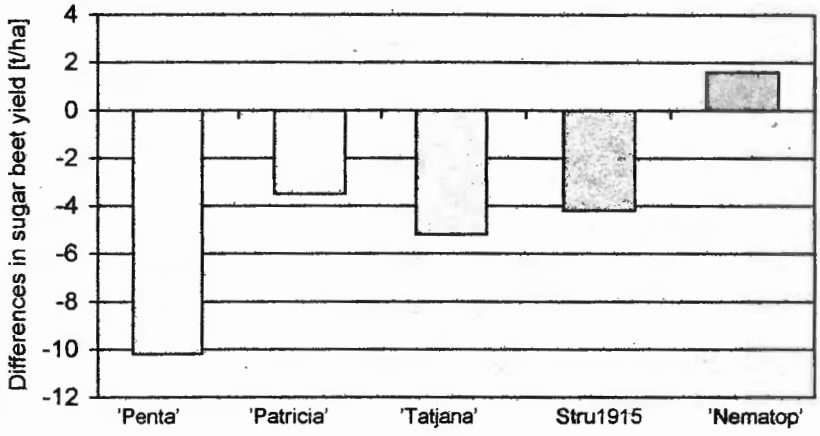

Figure 1. Differences in sugar beet yield of intolerant and tolerant cultivars and hybrids grown at low (540 E+J/100 ml soil) and high (3389 E+J/100 ml soil) initial densities (P_i) of *Heterodera schachtii* in 1999.

To better demonstrate tolerance, the relationship between increasing nematode densities and sugar beet yield is given for the three cultivars/hybrids 'Penta' (I-S), Stru1915 (T-PR) and 'Nematop' (T-R) in 1999 and 2000. In 1999 increasing nematode densities caused a rapid decrease in yield of the intolerant-susceptible cultivar 'Penta' (I-S) (Fig. 2). The yield decrease was less pronounced for the tolerant-partial resistant hybrid Stru1915 and no yield decrease was observed for the tolerant-resistant cultivar 'Nematop'. In 2000 all three cultivars/hybrids responded to increasing nematode levels with yield losses (Fig. 3). However, yield losses were highest for the intolerant-susceptible cultivar 'Penta' and lowest for the tolerant-resistant cultivar 'Nematop'.

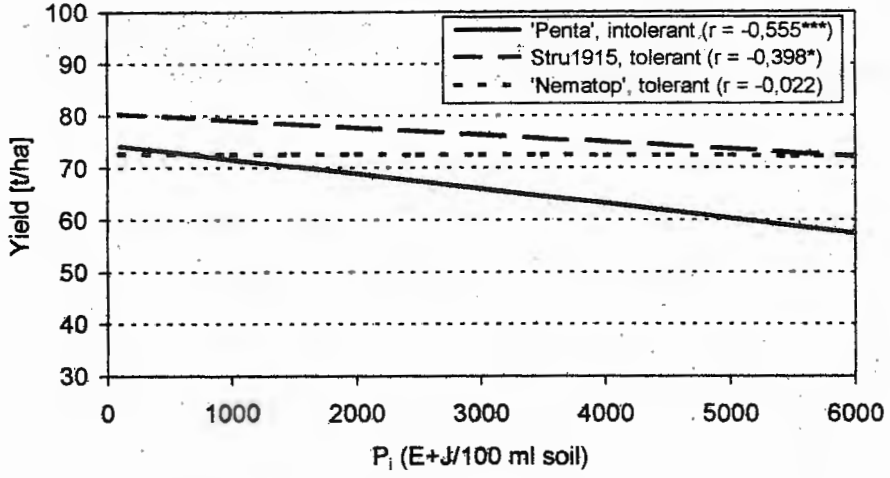

Figure 2. Relationship between increasing nematode densities of *Heterodera schachtii* and yield of intolerant and tolerant sugar beet cultivars and hybrids in 1999.

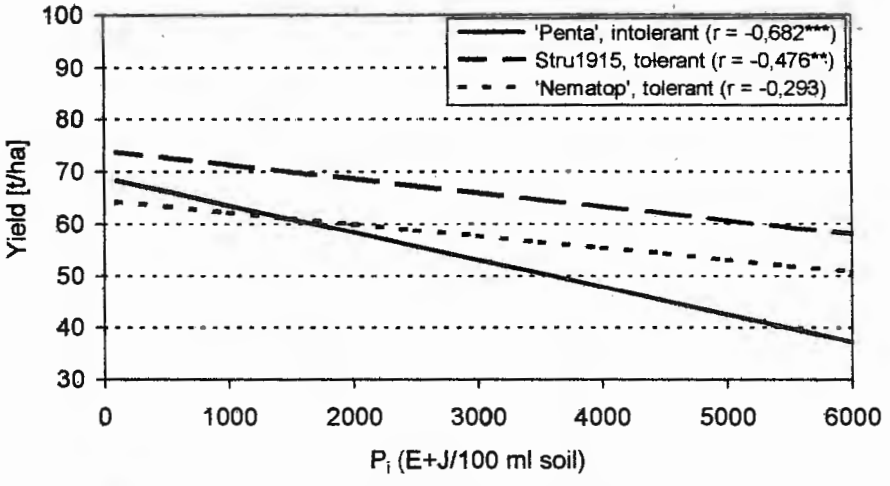

Figure 3. Relationship between increasing nematode densities of *Heterodera schachtii* and yield of intolerant and tolerant sugar beet cultivars and hybrids in 2000.

Greenhouse studies

In the experiment designed to study the relation of tolerance to rooting depth, shoot fresh weight and total root length, all three parameters were significantly higher for the tolerant-resistant cultivar 'Nematop' than for the intolerant-susceptible cultivar 'Penta' (Table 1).

Although total root length of both cultivars was negatively influenced by *H. schachtii* infestation, differences were more pronounced for the intolerant-susceptible cultivar 'Penta'. The highest level of root growth measured in cm length was always found in the top 10 cm and decreased with increasing depth (Fig. 4). In the absence of *H. schachtii* root length in the top 10 cm was 76 cm for 'Penta' (I-S) and 191 cm for 'Nematop' (T-R) while in 40-50 cm depth no roots were found for 'Penta' (I-S) but 20 cm root length were measured for 'Nematop' (T-R). Presence of *H. schachtii* significantly reduced rooting depth of both sugar beet cultivars. However, root growth of the intolerant-susceptible cultivar 'Penta' was restricted to the top 10 cm, while root growth of the tolerant-resistant cultivar 'Nematop' reached a depth of 40 cm.

Table 1. Shoot fresh weight and total root length of the intolerant-susceptible sugar beet cultivar 'Penta' and the tolerant-resistant cultivar 'Nematop' in the absence and presence of *Heterodera schachtii*.

	Shoot fresh weight [g]		Total root length [cm]	
	Control	*H. schachtii*	Control	*H. schachtii*
'Penta'	0,53	0,14	116	21,3
'Nematop'	1,01	0,45	275	147,4

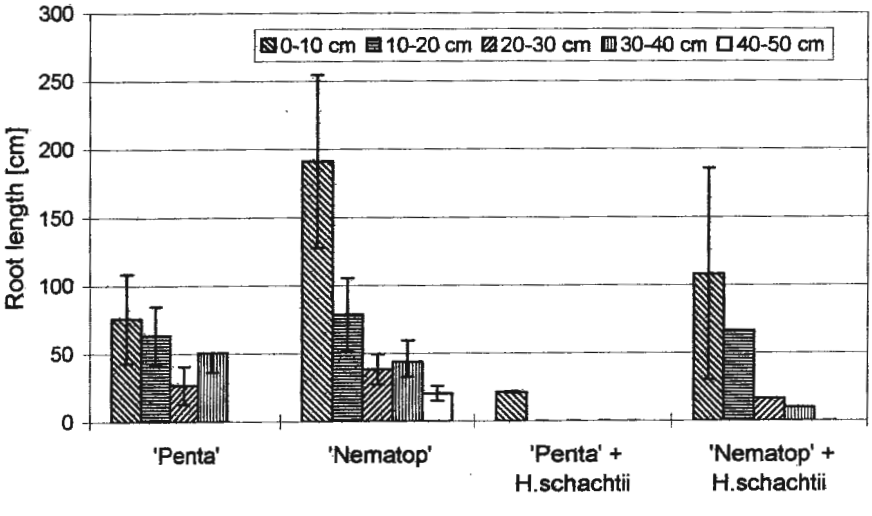

Figure 4. Root length of the intolerant-susceptible sugar beet cultivar 'Penta' and the tolerant-resistant cultivar 'Nematop' in relation to rooting depth and infestation with *Heterodera schachtii*.

Regarding compensatory growth, mechanical removal of 50 % of the tap root reduced shoot fresh weight of both cultivars (Table 2). However, reduction in shoot fresh weight was higher for the intolerant-susceptible cultivar 'Penta' (- 13 %) than for the tolerant-resistant cultivar 'Nematop' (- 6 %). Significant root length compensatory growth was observed for the tolerant-resistant cultivar 'Nematop' but not for the intolerant-susceptible cultivar 'Penta'. Root length of 'Nematop' increased from 488 cm in the control to 710 cm (+ 45 %) following treatment while for the intolerant-susceptible cultivar 'Penta' root length was reduced from 543 cm in the control to 445 cm (- 18 %) in treated plants.

Table 2. Shoot fresh weight and total root length of the intolerant-susceptible sugar beet cultivar 'Penta' and the tolerant-resistant cultivar 'Nematop' following mechanical removal of 50 % of the tap root

	Shoot fresh weight [g]		Total root length [cm]	
	Control	Removal of 50 % of the tap root	Control	Removal of 50 % of the tap root
'Penta'	3,65	3,18	543	445
'Nematop'	4,19	3,94	488	710

Discussion

Tolerance of five sugar beet cultivars and hybrids was tested under field conditions over two consecutive years. In general, increasing P_i of *H. schachtii* densities caused a decrease in sugar beet yield. The degree of tolerance as measured by yield varied between the two years. However, the relationship remained the same: 'Nematop' was the most tolerant cultivar followed by the hybrid Stru1915, whereas 'Penta' was confirmed as intolerant towards *H. schachtii*. Differences in the expression of tolerance between the two years can probably be explained by different climatic conditions. The summer of 1999 was much drier than in 2000. As dry conditions favour nematode damage and limit plant growth, tolerance becomes an essential factor for yield production under these conditions. In cases where water supply is not a limiting factor, as in 2000, yield differences between tolerant and intolerant plants were less pronounced. Similar to the field studies, tolerance was also evident under greenhouse conditions. The tolerant cultivar 'Nematop' had a greater rooting depth in the presence of *H. schachtii* and significantly more compensatory growth than the intolerant cultivar 'Penta'. Furthermore, shoot fresh weight of the tolerant cultivar 'Nematop' was less effected by nematode infestation or mechanical damage. Therefore, shoot fresh weight and length as early indicator of plant tolerance, should be further explored. In summary, tolerance was shown to improve yield stability under nematode infestation by influencing root growth and root system architecture. Despite these positive effects, breeding for tolerance towards plant parasitic nematodes still receives little attention. Increasing nematode population densities must be considered when tolerant cultivars are planted. For nematode control, cultivars containing both, resistance and tolerance would be preferable. This condition is met by the newly released cultivars 'Nematop' and 'Paulina'. However, continuous growing of resistant crops bears the risk of pathotype selection. Therefore, alternating resistant with susceptible but tolerant sugar beets in a management scheme might reduce pathotype selection and still guarantees high yields under nematode infestation.

Acknowledgements

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Pasteuria penetrans and the integrated control of root-knot nematodes

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Abstract: The prospects of using *Pasteuria penetrans* within an IPM strategy for root-knot nematodes (*Meloidogyne* spp) are not always considered promising. This is because of the perceived difficulties associated with its mass-production and the specificity of populations. The methods of *in vivo* production can be optimised to produce sufficient spores for small-scale treatments in protected cropping and high value field crops. Matching root-knot nematode populations with aggressive *P. penetrans* populations is possible and use can also be made of plant resistance and cultural treatments to diminish nematode levels without influencing residual *P. penetrans* spore densities.

Key words: biological control, *Meloidogyne* spp..

What is *P. penetrans*, how is it mass-produced and then deployed in soil?

The notable feature of *P. penetrans* is the endospore; it persists in soil and is resistant to desiccation. The spore is not motile and only if a compatible juvenile nematode comes in contact with the spore does attachment occur: the subsequent growth of the parasite occurs only after the nematode enters the plant host. Its effectiveness as a control agent does depend on the density of spores in the soil, so methods of production and systems of increasing or augmenting the spore densities are critical.

Contriving the most efficient system of mass-production of spores depends on a good host plant management and a record of soil temperature (Stirling & Wachtel, 1980; Darban, 2003; Pembroke, 2003). Only with ideal conditions will spore numbers of $>2 \times 10^6$ per female nematode be achieved. If host plants can be inoculated with appropriate numbers of spore-encumbered juveniles spore productivity will be a function of size of host root system, optimum nematode invasion and sustained plant vigour over 10 weeks or longer depending on soil temperature. Effective deployment of spores of *P. penetrans* can depend on the method of preparation; applying spores contained in root powder (Stirling& Wachtel, 1980) can be unreliable (Pembroke, 2003). Recent resarch would suggest that methods of harvesting spore-filled cadavers from root-systems might be necessary to enable more efficient application and distribution of spores in soil.

Persistence in soil

Techniques for monitoring spore densities are not well developed and it is difficult to account for the fate of spores in soil. Persistence must be influenced by the organic content as well as the soil structure and the movement of soil water. Also, there is no knowledge of microfauna and microflora that might ingest or colonise spores. Only under dry laboratory conditions has it been demonstrated that spores within tomato roots will maintain viability for up to 10 years (Giannakou et al., 1997).

How to recognise *P. penetrans*?

Endospores are 3-5 microns diameter, readily observed by squashing infected female nematodes on a slide. Calibrating spore densities from suspensions prepared from plant root systems is less convenient and subject to greater errors than those derived directly from females. Estimation of spores from soil extracts is even less practicable. Until more sensitive techniques are developed, the best means of determining if spores are present in a soil is by bioassay with nematode juveniles. Recognition of the developing stages before sporulation is necessary when developing a schedule for *in vivo* mass-production.

Where is *P. penetrans* known to work?

Examples of natural suppression of root-knot nematodes by *P. penetrans* are uncommon; its presence could well be underestimated. Soil type can be a factor influencing the distribution of *P. penetrans,* however, evidence suggests that it can be effective in sandy soil such as in Florida (Chen & Dixon, 1998) and in loamy soils in Ecuador and Tanzania (Trudgill et al., 2000). Its occurrence in perennial crops suggests that natural suppression may develop where soil remains undisturbed (Stirling & White, 1982). At present, no such long-term controlled studies have been done. Suppression has been demonstrated in long-term field rotation studies (Chen & Dixon, 1998; Trivino & Gowen, 1996; Trudgill et al., 2000; Weibelzahl et al., 1996), the key to the success of these depended largely on the compatibility of the local *Pasteuria* with the predominant nematode(s). Better opportunities for improving the success under field conditions might be achieved if resistant or less suceptible host crop species are included in the rotation and also the use of nematicides and solarisation (Tzortzakakis & Gowen, 1994). In fields where there may be mixtures of root-knot nematode species and populations problems of specificity might be overcome by the introduction of mixtures of *P. penetrans* from different sources (Tzortzakakis et al.,1997).

Current Developments

Some progress has been made in mass production of *P. penetrans,* presumably through an *in vivo* system and a product is available from Nematech Co. Ltd in Japan. There is no information available on the method of production of this wettable powder formulation that contains 10^9 spores per g., nor is there any information on the origin of the product and if it is a composite of several *P. penetrans* populations. Some progress with *in vitro* culture has been reported by incorporating culture filtrates of the bacterium *Enterobacter cloacae* in a nutrient broth (Hewlett et al., 2002).

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Variation of disease severity of bottom rot in field-grown lettuce and possibilities of control

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Abstract: Field studies showed, that bottom rot on lettuce caused by *Rhizoctonia solani* is an increasing problem in commercial field-grown lettuce production in Germany. The objective were to study seasonal, temporal and spatial changes in incidence and severity of bottom rot on lettuce in a field naturally infested with *R. solani* over three seasons. Results should allow a risk assessment of disease incidence in dependence on weather conditions and support the decision on the application of biocontrol agents and fungicides. The use of fungicides was the main control strategy of bottom rot in the past. At present, no plant protection products are listed for application on lettuce and alternative control methods are not available. So the effectiveness of known biocontrol products such as Prestop®, Phytovit® and FZB24® were evaluated against *R. solani*.

Key words: *Rhizoctonia solani, Lactuca sativa*, biocontrol, disease severity, field conditions

Introduction

The soilborne pathogen *Rhizoctonia solani* Kühn, teleomorph *Thanatephorus cucumeris* (Frank) Donk, occurs throughout the world and causes diseases on many economic important plant species (Sneh et al., 1996). The fungus was first described by Julius Kühn on potato 1858. *R. solani* is a species complex and can divided into more homogeneous groups called anastomosis groups (AGs). To date 12 anastomosis groups are recognized, which show a certain degree of host-specificity (Carling et al., 2002). *R. solani* causes bottom rot disease of lettuce (*Lactuca sativa* L.). In Germany, yield losses through bottom rot increased in the last years. Typical symptoms first appear as small rust colored necrotic spots on leaf midribs and leaf parts in contact with soil.

The frequency of cultivation of the host plant and of susceptible crops in the rotation influence the incidence and severity of Rhizoctonia diseases (Hyakumachi and Ui, 1982). Lettuce is frequently cultivated in short rotation, e.g. once per year on the same soil. Losses of sugar beet yields were about 10% after successive crops compared to fields with a long rotation (Komoto et al., 1979). Monoculture has also been associated with the decline of certain diseases in the field (Hornby, 1990). But no detailed information exists about seasonal influence on disease incidence and severity of bottom rot on lettuce or on changes in the spatial distribution of the pathogen in fields. The objectives were to study seasonal, temporal and spatial changes of disease incidence and severity of *R. solani* on lettuce after successive cropping in a natural infected field with *R. solani*.

In the past bottom rot on lettuce was mainly controlled by the application of fungicides. At present, neither chemicals nor resistant cultivars are available and alternative control methods are lacking. The use of microorganisms with antifungal effects against *R. solani* in practice could be a control strategy. Thus, the aim of this study were to evaluate the

effectiveness of known biocontrol products such as Prestop®, Phytovit® and FZB24® against *R. solani* on field-grown lettuce.

Material and methods

Evaluation of disease incidence and severity

The experiment ran on a field, naturally infested with *R. solani*, over three seasons. Two lettuce crops were grown per year between May and October. The temperature was recorded 5 cm above and 5 cm below soil surface. Cultivation and harvest of plants was performed according to farming standards. This include the incorporation of all harvest residues into the soil. The field was divided into 12 beds (100 m long) with six rows each. Fertiliser was added based on a chemical analysis of the soil prior to each planting. The lettuce were harvested by hand six or seven weeks after planting. Diseased leaves were cut off and left on the soil and unmarketable lettuce heads were not removed from the field. These crop residues remaining after harvest were incorporated into the top soil.

The spatial and temporal changes of bottom rot over three seasons was assessed in total 216 units (24 plants). Disease severity (DSI) was rated in four categories on a scale from 1 to 7 (Kofoet et al., 2001) for the same units at each harvest time.

Evaluation of control products

The effectiveness of control products was evaluated on the same field after six lettuce crops. Lettuce plants (cv. Nadine) were treated with Prestop®, Phytovit®, FZB24 or a fungicide. Each treatment was carried out in seven replications in a randomised design. Lettuce plants were treated twice with the microbial products, seven days before and three days after planting. The plants were treated with the fungicide three days after, or three and ten days after planting. Disease severity was assessed at harvest.

Results and discussion

The number of plants with slight symptoms of bottom rot was highest at first crop and decreased rapidly in the following crops, whereas the percentage rate of plants with moderate and severe symptoms increased with repeated cropping (Fig. 1). In average the disease severity increased significantly already within the first two seasons, from 22.6 % in the first year to 30.0 % in the second year. Thus total crop losses increased within the three seasons.

Table 1. Coefficients of Variation (CV) of spatial distribution analysing of *Rhizoctonia solani* in vertical (columns) and horizontal (beds) lines.

Year/crop	CV between beds	CV between columns
1999 / 1	19.9	24.7
1999 / 2	23.2	13.9
2000 / 1	12.9	14.3
2000 / 2	14.1	7.6
2001 / 2	9.7	4.3

Lettuce plants with severe damages of bottom rot occurred more in patches in the first crop. The analysis showed high coefficients of variation in spatial distribution of DSI in the field as well as in horizontal (cultivation direction) and vertical direction in the first year (Tab. 1). Variation in spatial distribution of DSI decreased during successive cropping over three seasons. Seasonal changes in DSI correlated with mean daily soil temperatures during the cultivation time. Higher temperatures resulted in higher DSI.

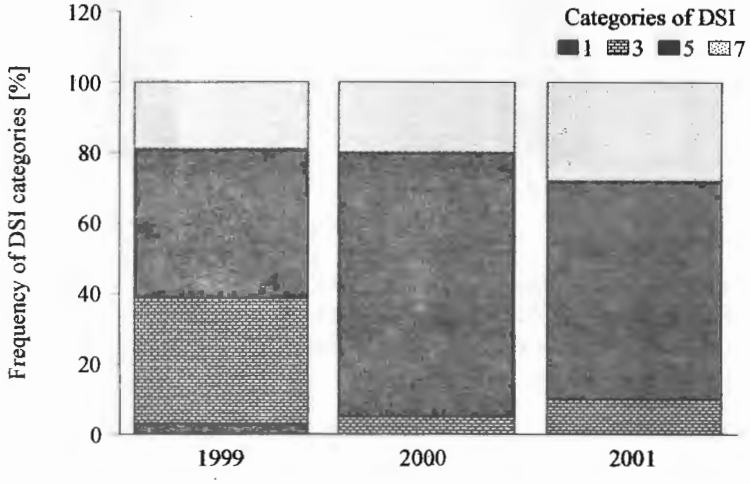

Figure 1. Frequency of rated disease severity index (DSI) categories (1 = healthy plants, 3 = low, 5 = moderate and 7 = heavy disease severity) of bottom rot on lettuce 'Nadine' in field.

The effectiveness of the biocontrol agents Prestop®, FZB24® and Phytovit® to control the causal R. solani on lettuce was tested in field experiment. Also the control effect of a fungicide in dependence of application schedule was checked. The number of lettuce plants with slight symptoms was significantly higher in all treatments compared to the control, excluded the treatment with Phytovit®. A significant lower number of plants with moderate symptoms was observed in the treatments with Prestop®, FZB24® and the fungicide. A significant reduction in disease severity was assessed in all fungicide treatments and in the treatment with Prestop® in comparison to the control (Fig. 2).

The fungicide and Prestop® have the potential to reduced the disease severity of bottom rot on lettuce. Further experiments have to prove, if the diseases are more suppressed by biological agents when repeatedly applied. Also is to evaluate, if a combination of a bacterial and a fungal biological agent, or a combination of a biological agent and a fungicide improve disease suppression.

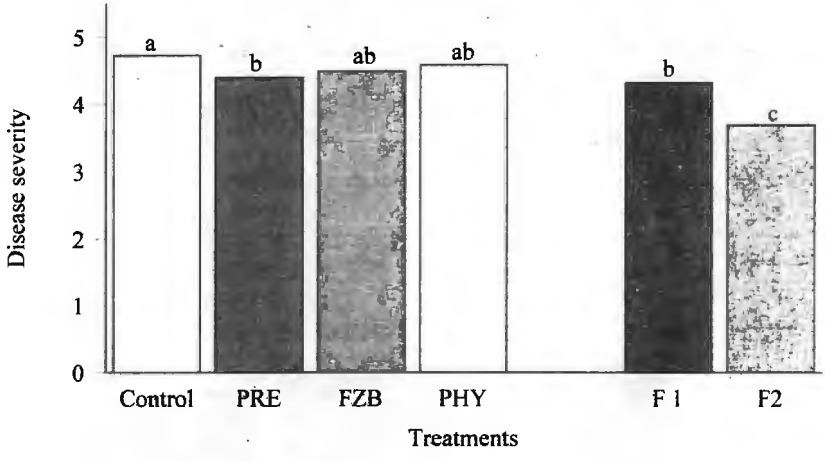

Figure 2. Effect of biological agents (Phytovit = PHY, FZB24 = FZB, Prestop = PRE) and a chemical on disease severity of lettuce 'Nadine' cultivated in a field, naturally infected with *Rhizoctonia solani*. The fungicide was applied once (F1) or twice (F2) after planting. [Kruskal-Wallis-test ($P<0.05$)].

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Endophytic bacteria and biological control of nematodes

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Abstract: Endophytic bacteria colonize the internal host tissue as do sedentary endoparasitic nematodes, which makes them potential candidates for biological control of nematodes. This review summarizes the work on endophytic bacteria in relation to biological control of nematodes. Isolation and screening procedures for antagonistic endophytes are described and bacterial candidates with biocontrol potential are presented. Their control mechanisms will be discussed as well as the nematode effects on bacterial population density and community structure. Possible advantages of endophytic bacteria in practical agriculture are pointed out.

Keywords: endophytic bacteria, plant-parasitic nematodes, biological control

Introduction

Plant parasitic nematodes can cause severe damage on most agricultural and horticultural crops. Especially the endoparasitic root-knot nematodes (*Meloidogyne* sp.) are among the most devastating nematodes. They are difficult to control due to their broad host range. Plant resistance is only available for few crops and chemical control measures are expensive and often restricted in use due to their high mammalian and environmental toxicity. In need of alternative control measures research on antagonistic bacteria as biological control agents has gained increasing interest. One such alternative is seen in antagonistic bacteria that colonize the same ecological niche inside the plant as the nematode, so called endophytic bacteria.

Although plants are colonized by a diverse spectrum of endophytic bacteria, still very little is known about their nature and function within the plant. Recent reports attribute several beneficial effects to endophytic bacteria, such as plant growth and plant health promotion. Few reports have also shown antagonistic activity of endophytic bacteria towards root-knot nematodes (Hallmann et al., 1995; Hallmann et al., 2001; Siddiqui & Shaukat, 2003).

Are endophytic bacteria the better biocontrol agents? So far, no product has been released and still many questions remain open such as for their ecological importance. What does the spectrum of naturally occurring endophytic bacteria look like and what proportion of these bacteria have antagonistic activity towards plant pathogens? How do they control plant parasitic nematodes? What are the underlying mechanisms? These are some of the questions that will be addressed in this review. However, the review also leaves some questions up to the future, such as what makes an endophyte to be an endophyte?

Endophytic bacteria

Endophytic bacteria are defined as those bacteria that can be isolated from surface-disinfected tissue or extracted from within the plant, and that do not visibly harm the plant (Hallmann et

al., 1997b). Basically, this definition includes culturable as well as non-culturable endophytic bacteria. However, since very little is known about non-culturable bacteria and the fact that they cannot be mass-reared for biological control purposes, this review focuses on culturable bacteria.

The first observations of bacteria residing in nonsymptomatic plants dates back to the 1870s (reviewed in Hollis, 1949). Within the following hundred years the existence of endophytic bacteria in different plant species was well documented for seeds (Mundt & Hinkle, 1976), tubers (Trevet & Hollis, 1948), roots (Philipson & Blair, 1957), stems, leaves (Henning & Villforth, 1940) and fruits (Samish et al., 1961). But it was not until the early 1990s that researchers were able to demonstrate beneficial effects of endophytic bacteria on plant growth and health (Chen et al., 1995; Dimock et al., 1988; Hallmann et al., 1995; Lalande et al., 1989; Pleban et al., 1995). At the same time, several chemicals were taken off the market for economical, toxicological and/or political reasons. Nematicides, which belong to the most toxic pesticides were especially affected and control alternatives had to be developed. The use of endophytic bacteria colonizing the same ecological niche as endoparasitic nematodes seemed to be a promising way to follow.

Isolation procedures

The isolation procedure is of key importance for working with endophytic bacteria. The proper procedure should recover all internal colonizers but no colonizers from the plant surface. In general, two approaches can be followed: 1. surface disinfestation followed by maceration of the plant tissue (Bell et al., 1995; Gardner et al., 1982; Hallmann et al., 1997a,b) and 2. extraction of the apoplast fluid by means of vacuum and pressure extraction or centrifugation (Dong et al., 1994). Both approaches have their strength and limitations. The first approach considers all internal colonizers, however, bacteria can avoid surface disinfection by hiding beneath collapsed root hairs or in fine niches between epidermal cells causing false positives. Furthermore, the surface disinfectant penetrates over time into the root tissue and the concentration and incubation time required to kill the most resistant bacterium on the outside might already be lethal to susceptible colonizers within the plant tissue. The second approach is somewhat limiting in that mainly bacteria of the vascular sap will be considered but not those colonizing the apoplast of the root cortex. However, depending on the research question, this can also be a desirable effect.

Most work on endophytic bacteria used surface disinfestation procedures for bacterial isolation. Depending on plant species, tissue and age, the optimum method differs in pretreatment, type of disinfectant and incubation time and needs to be experimentally adjusted for each scenario. Some generally used disinfectants include sodium hypochlorite (Gardner et al., 1982; Quadt-Hallmann et al., 1997), ethanol (Dong et al., 1994), hydrogen peroxide (McInroy & Kloepper, 1994) and mercuric chloride (Hollis, 1951). To reduce surface tension of the disinfectant detergents like Tween 20 (Hallmann et al., 1997b), Tween 80 (Sturz, 1995) or Triton X-100 (Mishagi and Donndelinger, 1990) have been added. Following surface disinfestation, the plant tissue is washed several times in sterile water. Sterility checks are included to recognize incompletely disinfected plant tissue which will be discarded. The plant tissue is than macerated using mortar and pestle or a mechanical device such as blender or a KLECO tissue pulverizer and streaked on nutrient agar for bacterial growth. Developing bacterial isolates are brought into clean culture and screened for beneficial effects.

Beneficial effects of endophytic bacteria

Plants provide nutrients and protection for endophytic bacteria, but do they also benefit from bacterial colonisation? Numerous reports over the past two decades have shown that

endophytic bacteria can significantly contribute to plant growth and plant health promotion (summarized in Hallmann et al., 1997b). According to Sturz (1995) approximately 10 % of bacterial isolates recovered from potato tubers showed plant growth promotion. Similar effects are expected for other plant/endophyte associations. Plant growth promotion can be facilitated by several microbial processes such as nitrogen fixation, nutrient solubilisation and production of plant growth hormones. N-fixation by free living endophytic bacteria has been described for bacterial genera such as *Acetobacter*, *Azoarcus*, *Azospirillum*, *Enterobacter* and *Klebsiella*, while production of plant growth regulators such as ethylene, auxins and cytokinins have been found in some strains of *Azospirillum*, *Azotobacter*, *Enterobacter*, *Pseudomonas* and *Staphylococcus* (summarized in Hallmann et al., 1997b). Hurek et al. (1994) reported, that inoculation of rice with *Azoarcus* sp. strain BH72 promoted plant growth, but growth promotion also occurred with *nif*$^-$ mutants, indicating that N_2 fixation by *Azoarcus* was apparently not involved in plant growth promotion. Other mechanisms such as enhanced plant mineral uptake and improved plant water relationship associated with endophytic colonisation might explain the observed effects.

Most work on plant health promoting effects by endophytic bacteria has been targeting bacterial and fungal pathogens (summarized in Hallmann, 2001). Several endophytic bacteria have shown control potential against soil-borne and wilt inducing pathogens. But how large is the proportion of indigenous bacteria with antagonistic properties? Krechel et al. (2002) studied this topic for potato-associated bacteria. Over two years and at three sampling times per year (youth, flowering, senescence) they characterized the bacterial spectrum of the four microhabitats rhizosphere, endorhiza, phyllosphere and endosphere. The antagonistic potential of > 2,500 bacterial isolates was evaluated as antibiosis towards *Verticillium dahliae* and *Rhizoctonia solani*. Within this scope, 9.2 % of all bacteria showed antagonistic potential (Table 1). Throughout the season, the antagonistic potential started low with 5.5 % at youth stage, increased to 13.2 % at flowering and finally decreased to about 8.9 % at senescence. Regarding the microhabitats, the highest antagonistic potential was found in the endorhiza with 13.3 % followed by the rhizosphere with 11.8 %. These are the microhabitats where soil-borne pathogens and plant parasitic nematodes attack the plant and it seems that the plant establishes a first defence line towards these pathogens by supporting antagonistic bacteria within these microhabitats. Compared with the rhizosphere and endorhiza, the antagonistic potential in the above-ground microhabitats was lower with 8.4 % in the phyllosphere and 3.3 % in the endosphere. The highest single occurring antagonistic potential of 21 % was found for the endorhiza at flowering.

Table 1: Proportion of potato-associated bacteria expressing antibiosis towards *Verticillium dahliae* and *Rhizoctonia solani*

Growth stage	Rhizosphere	Endorhiza	Phyllosphere	Endosphere	Total
Youth	10.2	4.0	3.4*	4.4	5.5
Flowering	11.6	21.0	18.4	1.7	13.2
Senescence	13.5	15.0	3.3	3.8	8.9
Total	11.8	13.3	8.4	3.3	**9.2**

*data only for one year

Biological control of plant parasitic nematodes

As shown above, endophytic bacteria can significantly improve plant growth and plant health. But do they also control plant parasitic nematodes? Plant parasitic nematodes seem to be a more difficult target for endophytic bacteria than fungal or bacterial pathogens. They are robust organisms and their stylet allows them intra- and intercellular movement through the plant tissue thus limiting the efficacy of endophytic antagonists. However, sedentary endoparasites such as root-knot nematodes (*Meloidogyne*) and cyst nematodes (*Heterodera*, *Globodera*), which stay localized within the plant for several weeks and feed from a single feeding site might be an interesting target for endophytic bacteria.

Screening endophytic bacteria for antagonism towards plant parasitic nematodes is mostly done on host plants and therefore means a time-consuming process. *In vitro* testing for anti-nematode substances as being done for rhizosphere bacteria (Becker et al., 1988) is possible, but the fact that those substances will be expressed in the plant can be an undesired attribute when it comes to commercialisation. To avoid blind testing of thousands of endophytic bacteria, criteria for the preselection of bacteria with high chances of antagonistic activity need to be developed. Possible criteria could be: 1) known biocontrol agents with antagonistic activity towards other plant pathogens, 2) isolates of bacterial species with high antagonistic properties such as *Pseudomonas* and *Bacillus*, 3) isolates maintained from a healthy plant among infested plants, 4) endophytic bacteria isolated from plants grown in suppressive soil, 5) endophytic bacteria isolated from nematode antagonistic plants or 6) endophytic bacteria with certain physiological traits such as chitinolytic activity.

Following preselection, Hallmann et al. (1996) found an average of 38 % of all bacterial groups to express antagonistic activity towards *M. incognita* in a first screening step (Table 2). The best results were achieved for the genus *Serratia* and for endophytic bacteria expressing chitinolytic activity with 50 % and 47 % of the isolates significantly reducing gall index or number of galls and/or egg masses of *M. incognita*. From 25 endophytic bacteria with antagonistic activity towards *M. incognita* on cotton, ten isolates (40 %) showed chitinolytic activity, 16 isolates (64 %) showed proteolytic activity and 13 isolates (52 %) produced culture filtrates inhibitive to *M. incognita* juveniles at 50 % and more.

Table 2: Effect of bacterial sources on the proportion of endophytic bacteria with antagonistic activity towards *Meloidogyne incognita* on cotton and cucumber

Source	
known antagonists	30 % (20)*
untreated soil	38 % (16)
chitin-treated soil	27 % (15)
chitinolytic activity	47 % (19)
Genera	
Serratia	50 % (10)
Burkholderia	33 % (15)
Physiological traits	
chitinase activity	40 % (25)
protease activity	42 % (38)
In vitro activity of bacterial culture filtrates from nematode antagonistic endophytes	45 % (33)

*total number of bacterial isolates tested is given in parenthesis

The majority of the still little work on biocontrol has been targeting *M. incognita*, one of the economically most important nematode species worldwide. Nothing is known about potential effects towards ectoparasitic nematodes and/or virus transmission by dorylaimid nematodes. Table 3 lists nematode species, bacterial endophytes and host plants where significant control of plant parasitic nematodes was achieved. The control spectrum of one particular endophyte can be narrow or very broad as being shown for *Rhizobium etli* G12. *R. etli* G12 significantly controlled root-knot nematodes as well as cyst and root lesion nematodes on several host plants (Mahdy et al., 2001).

Table 3: Control of plant parasitic nematodes by endophytic bacteria

Nematode species	Bacterial endophyte	Host plant	Reference
Meloidogyne incognita	*Brevundimonas vesicularis*, *Burkholderia cepacia*, *Cedecea davisae*, *Pantoea agglomerans*, *Phyllobacterium rubiacearum*, *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida*, *Rhizobium etli*,	cotton, cucumber, soybean, bean, pepper, potato	Hallmann et al., 1997c, 1998, 1999, 2001; Mahdy et al., 2001; Munif et al., 2001; Siddiqui & Shaukat, 2003
Meloidogyne javanica	*Pseudomonas aeruginosa*, *P. fluorescens*, *Rhizobium etli*	mungbean, soybean, tomato	Mahdy et al., 2001; Siddiqui & Shaukat, 2003
Globodera pallida	*Rhizobium etli*	potato	Mahdy et al., 2001
Heterodera schachtii	*Rhizobium etli*	sugarbeet	Mahdy et al., 2001
Pratylenchus zeae	*Rhizobium etli*	maize	Mahdy et al., 2001

However, as interest in the antagonistic potential of endophytic bacteria towards plant parasitic nematodes is increasing, more and more reports confirm the broad occurrence of antagonistic endophytes. Several plant-health promoting rhizosphere bacteria, once looked properly, turned out to also colonize the root cortex. Kloepper et al. (1992) have shown, that five of six rhizobacteria, which induced systemic resistance in cucumber, exhibited both external and internal root colonisation (Kloepper et al., 1992). Exploiting an additional microbial habitat for biocontrol purposes might enhance overall disease control and increase control consistency, since the control agent could avoid unfavourable conditions in one habitat by escaping into the other habitat.

Although some endophytic bacteria have shown excellent control of plant parasitic nematodes, control is often not consistent and does seldom exceed 50 %. Consistent control has been shown for *Pseudomonas fluorescens* 89B-61 (Hallmann et al., 1998), *Brevundimonas vesicularis* IN884, *Serratia marcescens* 90-43 (Hallmann et al., 1997c) and *Rhizobium etli* G12 (Magdy et al., 2001) against *M. incognita*. However, most experiments were conducted in the greenhouse and more field data are needed to confirm the biocontrol potential of endophytic bacteria under practical conditions.

Mode of action

In general, endophytic bacteria can control plant parasitic nematodes by either 1) pre-emptive colonisation, 2) direct antagonism by anti-nematode substances and 3) induced resistance. Pre-emptive colonisation, i. e. using endophytic species colonizing the same ecological niche as the pathogen, might work for closely related species such as non-pathogenic *Fusarium oxysporum* to control pathogenic forms of *F. oxysporum*, *Agrobacterium radiobacter* K84 to control *A. tumefaciens* or *Enterobacter* sp. to control *Erwinia amylovora* but for reasons mentioned earlier seems to be less likely to account for nematode control. Bacteria-derived anti-nematode substances, also of high effectivity, are less suited for biocontrol agents since they can prevent later registration. The mode of action presently gaining most interest is the induction of plant defence mechanisms.

In general, induced resistance has been shown for many endophytic bacteria controlling bacterial and fungal pathogens (summarized in Hallmann, 2001). More recently, induced systemic resistance was identified as control mechanism of *Rhizobium etli* G12 against *Globodera pallida* (Hasky-Günther et al., 1998), *Pantoea agglomerans* MK-29, *Cedecea daviasae* MK-30, *Enterobacter* sp. MK-42 and *Pseudomonas putida* MT-19 against *M. incognita* (Munif et al., 2001) and *Pseudomonas aeruginosa* IE-6S+ against *M. javanica* (Siddiqui & Shaukat, 2002). All these studies were done in split-root systems where one half of the root was treated with the bacterium and the other half with the nematode. While induction of plant defence mechanisms requires some kind of plant/endophyte recognition, the hypothesis might be raised, that endophytic bacteria are better inducers than rhizosphere bacteria since they establish a much closer relationship with the plant.

The origin of the inducing agent has been intensively studied for *R. etli* G12. Based on the work by Hasky et al. (1998) and Reitz et al (2000) it can be concluded that lipopolysaccharides (LPS) of the bacterial surface function as elicitor. The LPS supposedly binds to receptors on or near the plant cell surface inducing the defence response. This mechanism is supported by work on LPS preparations of other bacteria such as *Xanthomonas campestris*, *Pseudomonas solanacearum*, *P. fluorescens* and *P. putida* inducing resistance towards other plant pathogens (summarized in Hallmann, 2001). Comparing *R. etli* G12 with the rhizosphere colonizer *B. sphaericus* B43 viable and heat-killed cells of both bacteria triggered the defense reponse, as did culture filtrates of *B. sphaericus* B43 but not of *R. etli* G12 indicating differences in the mode of action between these two bacteria.

Population density

If induced resistance is the possible mode of action, what population densities are required to induce this response. What population sizes do they reach and how is that affected by plant parasitic nematodes? Summarizing the literature, population densities of bacterial endophytes seem to be highest in the root with about 10^5 cfu/g fresh weight. Moving acropetal, population densities generally decrease to 10^4 cfu/g fresh weight in the stem and about 10^3 cfu/g fresh weight in leaves (Hallmann et al., 1997b). The lowest population densities are found in generative plant parts such as flowers, ovules and seeds. The fact that above-ground populations densities are lower than below-ground populations could be an indicator for less favourable conditions in above-ground plant parts. Daily fluctuations in temperature as well as water und nutrient availability are higher above-ground than below-ground.

For the root system populations densities of 10^5 cfu/g fresh weight properly seem to represent the maximum holding capacity for endophytic bacteria. Attempts to increase the population density above 10^5 cfu/g by creating wounds to promote bacterial entrance or by injection of a bacterial suspensions into the root did not succeed.

However, plant parasitic nematodes do increase population densities of endophytic bacteria (Hallmann et al.,1998). For example, with increasing nematode inoculum of *M. incognita* from 0 to 3000 juveniles, the internal population density of the introduced endophyte *Enterobacter asburiae* JM22 increased from 10^2 cfu/g to about 10^5 cfu/g root fresh weight. Similar relationships can be expected for antagonistic endophytes. This phenomenon can be most likely explained by wounds created by the nematode which than provide entry avenues for the bacteria, and by leakage of plant nutrients which serve as bacterial nutrients thus supporting higher bacterial populations which than can enter the root tissue. However, individual bacterial cells can also attach to the nematode cuticle and being carried by the nematode into the plant tissue (Hallmann et al., 1998). Dose response studies on *R. etli* G12 indicated 10^8 cfu/ml as minimum bacterial densities to receive significant control of *M. incognita* on tomato (Hallmann, unpublished). For the endophytic bacterium *P. aeruginosa* an application rate of more than 2.5 x 10^8 cfu/ml did not further promote biocontrol of *M. javanica* but concentrations below this level significantly reduced nematode control (Siddiqui & E' teshamul-Haque, 2001).

Bacterial spectrum

The spectrum of endophytic bacteria depends on numerous biotic and abiotic factors that often interact. In general, Gram-negative bacteria are dominating the endophytic spectrum. They accounted for 78 % of the strains isolated from grapevine xylem (Bell et al., 1995), 84 % of the strains isolated from citrus roots (Gardner et al., 1982) and 100 % of the strains from cotton roots (Hallmann et al. 1999). The most common genera are *Pseudomonas*, *Burkholderia*, *Phyllobacterium* and *Enterobacter* (summarized in Hallmann et al. 1997b; Hallmann, 2001) For Gram-positive endophytes, *Bacillus* is probably the most widely distributed genus. For most of these genera antagonistic isolates have been reported.

The presence of plant parasitic nematodes not only increases total numbers of endophytic bacteria (Hallmann et al., 1997b) but also changes their community structure. In comparative studies on cotton differences in the bacterial spectrum between noninoculated plants and plants inoculated with *M. incognita* were seen within seven days after nematode inoculation (Hallmann et al., 1998). For example, *Brevundimonas vesicularis* was predominant in noninoculated plants (12.6 %) compared to inoculated plants (4.0 %) and *Burkholderia pickettii* (8.6 %) and *Alcaligenes xylosoxydans* (4.7 %) were exclusively isolated from inoculated plants. *Agrobacterium radiobacter* and *Pseudomonas* spp. were consistently isolated from both noninoculated (21.9 % and 18.7 %) and inoculated (18.6 % and 18.8 %) plants. Overall, endophytic bacteria recovered from cotton roots inoculated with *M. incognita* showed a higher diversity based on bacterial richness and diversity, but differences were not significant (Hallmann et al., 1998).

Mode of entry

One way of entering plants has been described for endopyhtic bacteria above using nematodes as vector. Probably the simplest way to colonise the plant have seed-borne endophytes, since they are already in the plant. Although endophytic bacteria have been detected in and isolated from seeds (Mundt & Hinkle, 1976; Pleban et al., 1995), the complete passage from seed to seed has not yet been shown. This leads to the more favoured hypothesis that plants are primarily colonised from the outside such as from the rhizosphere or phyllosphere. Major routes for bacterial entrance are: 1) natural openings (stomata, hydathodes, lenticels), 2) wounds caused by pathogens, soil abrasion, formation of lateral roots or abiotic factors, and 3) micropores (Hallmann, 2001). However, plant openings are not absolutely required for bacterial entrance. Seedlings grown with minimal disturbance in liquid media or water agar

were colonised by endophytic bacteria long before lateral roots were formed (Levanony & Bashan, 1989; Quadt-Hallmann et al., 1997b). Further investigations indicated that colonisation by endophytic bacteria can be associated with cellulytic and pectinolytic enzymes (Hurek et al., 1994; Quadt-Hallmann et al., 1997b). Quite interestingly, enzymatic degradation of plant cell walls by these bacteria was only observed during the colonization process but never after the bacteria had established in the root cortex.

After gaining entrance, endophytic bacteria either remain localised in the specific plant tissue or they colonise the plant systemically. Systemic colonisation by endophytic bacteria has been shown for intercellular spaces of 15 cells (Hurek et al., 1994) to distances representing 1-4 cm (Mahaffee et al., 1997). *Enterobacter asburiae* JM22 applied as a seed treatment was reisolated from cotton leaves within few days after application (Quadt-Hallmann et al., 1997b). But does the colonization habit can affect biocontrol efficacy? For endophytic antagonists with direct mode of actions like parasitism or toxin production, systemic colonization might be an important trait to ensure contact with the nematode. However, bacteria releasing toxins into the plant or colonizing the harvest organs are not acceptable as biocontrol agents. Therefore, local colonisation is the preferred attribute. Quite luckily, endophytic biocontrol bacteria studied so far turned out to be local colonizers, such as *Pseudomonas fluorescens* 89B-27, P. *fluorescens* 89B-61 and *R. etli* G12 (Hallmann, 2001; Hallmann et al., 2001; Quadt-Hallmann et al., 1997b) while on the other side intensive testing of the excellent systemic colonizer *E. asburiae* JM22 against a broad spectrum of viral, bacterial, fungal and nematode pathogens showed no effect (Hallmann, unpubl.). However, to associate colonisation patterns with strategies of biological control agents we need to know more about bacterial localisation.

Localization

Endophytic bacteria have been isolated from various plant parts. But where are they exactly located within these plant parts? Do they preferably occur in the symplast or apoplast? Are they homogenously distributed throughout the plant tissue? Modern detecting methods using serological, molecular and microscopic approaches have gained some excellent insight in bacterial localisation. In general, endophytic bacteria can colonise both the intercellular and intracellular space, however, intercellular colonisation represents most likely the dominant form. An excellent tool for studying bacterial colonisation in plant tissue proved to be bacterial marking with the green fluorescence protein (GFP) in combination with confocal laser scanning microscopy. As shown by Hallmann et al. (2001) transformation of the endophytic antagonist *R. etli* G12 with pGT-trp containing a *trp-gfp* transcriptional fusion expressed high levels of GFP. The plasmid was quite stable inherited and genetically transformed bacteria showed no differences in colonisation behaviour and biological control potential compared with their wild-type. But not all plant roots are suited for confocal laser scanning microscopy, since the opacity of the root can make observations of bacteria in deeper root tissue impossible. Within this respect, *Arabidopsis thaliana* with its hyaline roots represents an excellent model plant.

The GFP-marked endophytic antagonist *Rhizobium etli* G12, designated as G12(pGT-trp), colonized the root tissue of *Arabidopsis* intra- and intercellularly occurring in epidermal cells, root cortex and vascular tissue (Hallmann et al., 2001). Colonisation by *R. etli* G12(pGT-trp) was most pronounced in the intercellular space of the root cortex, where colonisation sites were heterogeneously distributed. Within the vascular system microcolonies were observed which formed either a discontinuous or a more continuous chain. Regarding intracellular colonisation, *R. etli* G12(pGT-trp) was observed in individual epidermal cells including root hairs while adjacent cells were not colonized. Cells and intercellular spaces

colonised by *R. etli* G12(pGT-trp) were usually tightly packed with bacteria and bacterial movement within the cells was clearly visible. Given the large number of small and discontinuously colonised sites, colonisation might result from 1) occasional bacterial invasion of the root and efficient colonization of certain cells that bacteria come into contact, or 2) frequent entry of bacterial cells into a root, their movement through the root but infrequent penetration of root cells, or both.

In *Arabidopsis* roots inoculated with *M. incognita*, large numbers of *R. etli* G12(pGT-trp) were found at the nematode penetrations sites and within galled root tissue. Approximately 20 % of the nematode galls were colonised by *R. etli* G12(pGT-trp) and colonised galls seemed to be randomly distributed throughout the root system. The close proximity of *R. etli* G12(pGT-trp) with *M. incognita* can probably be attributed to the availability of nematode-derived nutrients that become available to the bacterium. Leakage of nutrients occurs at nematode penetration sites and is probably also the case within nematode galls where the nematode-induced feeding site represents a metabolic sink containing four to six times more glucose and free amino acids than the actively growing root tip cells (Huang, 1985). However, nutrient competition as potential mechanisms for nematode suppression is doubtful as long as there is no evidence that bacteria actively derive nutrients from the nematode feeding site. Taken into account the heterogeneously distribution of endophytic bacteria within the root tissue, other mechanisms such as induced resistance are mor likely to occur (see above).

Conclusions

Endophytic bacteria with antagonistic activity against plant parasitic nematodes might be excellent biocontrol agents for the following reasons: 1) mass production in fermenters possible, 2) simple application via seed treatment, 3) internal plant tissue provides a uniform and protective environment for the bacteria, and 4) less competition due to fewer types and numbers of organisms. In practical agriculture, control efficacy and consistency might even be improved by combining bacterial endophytes with rhizosphere and foliar antagonists, leading to a holistic biological control system that works against multiple pathogens of both root and foliar tissue. However, endophytic bacteria also have to face some challenges like acceptability by the host plant, rapid and competitive colonisation of the young seedling and enhanced biocontrol efficacy. Since the potential uses of endophytic bacteria in agriculture are manifold, further research is urgently needed to develop endophytic bacteria to a practical application.

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Micro-organisms and broadspectrum induced systemic resistance

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Abstract: Root knot nematodes (*Meloidogyne incognita*) and the wilt disease caused by the soilborne fungus *Fusarium oxysporum* f.sp. *lycopersici* alone or in combination cause severe losses in tomato production world-wide. As an alternative to chemical soil fumigation, we selected five different rhizobacteria strains that are able to reduce wilt symptoms caused by *F. oxysporum*, or the number of root galls caused by *M. incognita*. As an approach to optimize the effect of resistance-inducing rhizobacteria against root-knot nematodes, we tested combinations of different bacteria strains. The mode of action of these bacteria was tested in different experimental set-ups. Induction of systemic resistance was found to be the responsible mechanism. Induced changes in plant metabolism were assessed for rhizobacteria alone or in combination with *F. oxysporum*. Activities of enzymes related to defense against pathogens as Catalase, β-1-3-Glucanase, or Peroxidases were determined under different conditions. Phenol patterns, chlorophyll contents and cell wall structure were analysed. Transient alterations were found for Peroxidases. Changes were as well observed for phenols and chlorophyll. Plant responses differ between various rhizobacteria strains, even when resistance is induced against the same pathogen. These alterations are probably not alone responsible for reduced wilt symptoms or reduced nematode penetration. So other experimental approaches are needed to reveal mechanisms responsible for reduced wilt symptoms or nematode penetration. 1- and 2-dimensional protein gel electrophoresis and mRNA-based techniques present the method of choice.

Key words: Tomato, *Fusarium oxysporum* f.sp. *lycopersici*, *Meloidogyne incognita*, induced systemic resistance, rhizobacteria.

Introduction

Soilborne diseases like *Fusarium* wilt or root galls, caused by *Fusarium oxysporum* f.sp. *lycopersici* and *Meloidogyne incognita*, respectively, present a serious thread for tomato production in tropical and subtropical regions. Until now, these diseases were controlled by chemical soil fumigation. Methyl bromide, the most effective and therefore most commonly used soil fumigant will be banned in a near future because of its nocious effects on atmospheric ozon (Robert 2001). World-wide, different efforts are produced to find alternatives to chemical soil fumigation. Among these, biological control using rhizobacteria presents a promising perspective to control soilborne diseases.

Biological control of *Fusarium oxysporum* f.sp. *lycopersici* using bacteria has been studied for many years (Weller, 1988). Rhizosphere or root colonizing bacteria and fungi were used most frequently as biological control agents of *Fusarium* (Chen et al., 1995). Among bacteria, *Pseudomonas* is the most frequent genus used to control *Fusarium* (Fuchs and Défago, 1991; Lemanceau and Alabouvette, 1991; Duijff et al., 1998) but *Bacillus* (Bochow and Dolej, 1998), and *Streptomyces* species are also important (Weller, 1988; Landa et al., 1997). Besides rhizobacteria, apathogenic strains of *Fusarium oxysporum* are as well able to control *Fusarium* wilt symptoms. Biological control of plant-parasitic nematodes by rhizobacteria was first reported by Becker et al. (1988). Subsequently, different bacteria

strains were characterized that reduce penetration of different nematodes (Racke and Sikora, 1992; Hoffmann-Hergarten et al., 1998; Neipp and Becker, 1999; Hauschild et al., 2000; Siddiqui et al., 2001).

Modes of action of biocontrol organisms present the central aspect in understanding and optimizing biological control. Among rhizobacteria controlling *Fusarium*, there are several antibiotic or siderophore producers that have a direct effect on the pathogen (Rosales et al., 1995; Landa et al., 1997). Competition for space especially on the root surface or competition for nutrients is another frequent mode of action (Fravel and Engelkes, 1994). Nematicidal substances produced by rhizobacteria can be responsible for biocontrol of nematodes (Becker et al., 1988).

Induction of systemic resistance is an indirect mode of action of biocontrol organisms, mediated by the plant. Resistance induction against *Fusarium* in tomato was reported for *Bacillus subtilis* FZB 24 (Bochow and Dolej, 1998) and *P. fluorescens* WCS 417r (Duijff et al., 1998). Two different rhizobacteria strains (*Rhizobium etli* G12 and *Bacillus sphaericus* B43) are able to induce systemic resistance against plant-parasitic nematodes (Hasky-Günther et al., 1998). Recently, resistance induction against *M. incognita* by a *Pseudomonas* isolate was reported by Siddiqui and Shaukat (2002).

In our group, different rhizobacteria were isolated and screened for their activity to reduce wilt symptoms caused by *F. oxysporum* f.sp. *lycopersici* or root galling caused by *M. incognita*. Five strains were found to be effective against one or both of the pathogens. The mode of action was determined in different *in vitro* tests and by separate application of the pathogen and the rhizobacteria. Induced systemic resistance against *Fusarium* was found to be responsible for three strains (Mwangi et al., 2001). Induced systemic resistance against *M. incognita* was found for one of these strain and two others.

Material and methods

Organisms used in this study

The rhizobacteria strain that were used are listed in table 1. They were isolated from the rhizosphere of different plants and originally tested against different nematodes on various host plants. Bacteria were grown in synthetic media (TSB or King's B for *R. etli* G12), centrifuged, resuspended in water and applied as soil drench. Details are given by Mwangi et al. (in preparation).

Table 1. Rhizobacteria strains used in this work

Isolate	Origin	known effect against	Reference
Bacillus sphaericus B43	Potato	*Globodera pallida*	Racke and Sikora, 1992
Bacillus thuringiensis Bt2	Tomato	*Meloidogyne incognita*	Terhardt, 1998
Pseudomonas fluorescens T58	Sugar beet	*Heterodera schachtii*	Oostendorp, 1986
Pseudomonas putida Pp53	Tomato	*F. oxysporum* f.sp. *lycopersici*	Terhardt, 1998
Rhizobium etli G12	Potato	*Globodera pallida*	Racke and Sikora, 1992

Tomato plants (*Lycopersicon esculentum* cv. "Hellfrucht Früher Stamm" and cv. "Rheinlands Ruhm") were used in the experiments. *Meloidogyne incognita* (race 3) were multiplied on tomato plants and extracted as described by Hussey and Barker (1973). Plants were inoculated

with juveniles or eggs. *Fusarium oxysporum* f.sp. *lycopersici* race 1 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, isolate DSMZ 62059). *F. oxysporum* was grown on PDA or in PDB liquid cultures. Spore suspensions were used to inoculate plants. Separation of rhizobacteria and *M. incognita* was achieved with split-root plants as done Hasky-Günther et al. (1998). *Fusarium* was separated from rhizobacteria by inoculating bacteria into the rhizosphere, and cutting the shoot and setting it into soil infested with *F. oxysporum* spores, similarly to the method described by Fuchs et al. (1997).

***Evalution of nematode penetration and wilt due to* Fusarium**

Nematode penetration was assessed as numbers of galls per plant or per root fresh mass. Egg masses were counted as a relative parameter for nematode reproduction. *Fusarium* wilt symptoms were classified using a wilt index from 1 to 5 as described by De Cal et al. (1999).

Results and discussion

Biocontrol efficiency of selected rhizobacteria

Tomato plants were inoculated with rhizobacteria and subsequently infested with *M. incognita* juveniles. Gall numbers were determined 7-8 weeks after nematode inoculation. The five rhizobacteria *Bacillus sphaericus* B43, *B. thuringiensis* 2, *Pseudomonas putida* 53, *P. fluorescens* T58, and *Rhizobium etli* G12 were able to decrease gall numbers and numbers of egg masses (Figure 1). The potential of the bacteria to reduce wilting caused by *F. oxysporum* was tested as well. Inoculation of three of the bacteria strains *B. sphaericus* B43, *P. fluorescens* T58, and *P. putida* 53 led to significant reductions in *Fusarium* wilt symptoms (Hauschild et al., 2001).

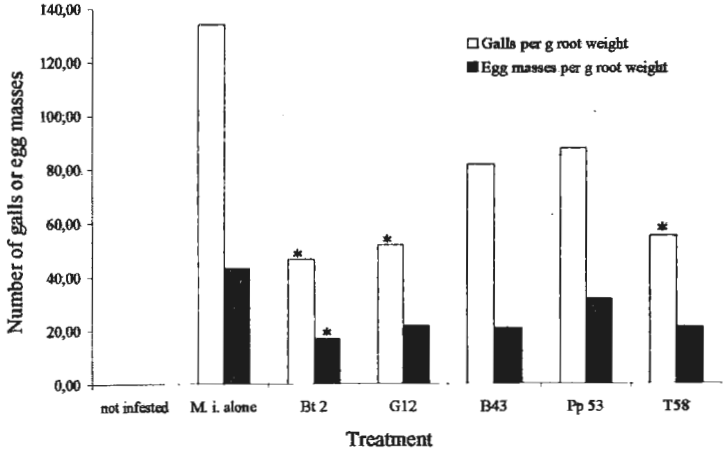

Figure 1. Numbers of *Meloidogyne incognita* galls and egg masses per root fresh weight. Three week old tomato plants were treated with rhizobacteria as a soil drench. 2700 *M. incognita* eggs per plant were inoculated three days later. Galls and egg masses were counted after 8 weeks. Treatments: not infested: no bacteria, no nematodes; M.i. alone: no bacteria treatment; Bt 2: *Bacillus thuringiensis* 2; G12: *Rhizobium etli* G12; B43: *Bacillus sphaericus* B43; Pp 53: *Pseudomonas putida* 53; T58: *Pseudomonas fluorescens* T58: Values with statistically significant differences (Dunnett T-test, $p \leq 0,05$; $n = 5$) compared to untreated infested plants are marked with an asterisk *.

Three of the strains that are effective in reduction of nematode penetration were tested in combinations. For all combinations tested, nematode penetration was reduced when compared to non-bacterized control plants, but also when compared to the plants treated with the bacteria alone (Figure 2). The additional effect of different isolates may be a hint to a different or complementary mode of action of these bacteria in reduction of nematode penetration. On the other hand, combination of different rhizobacteria strains may be a promising perspective in optimizing and stabilizing the biocontrol efficiency.

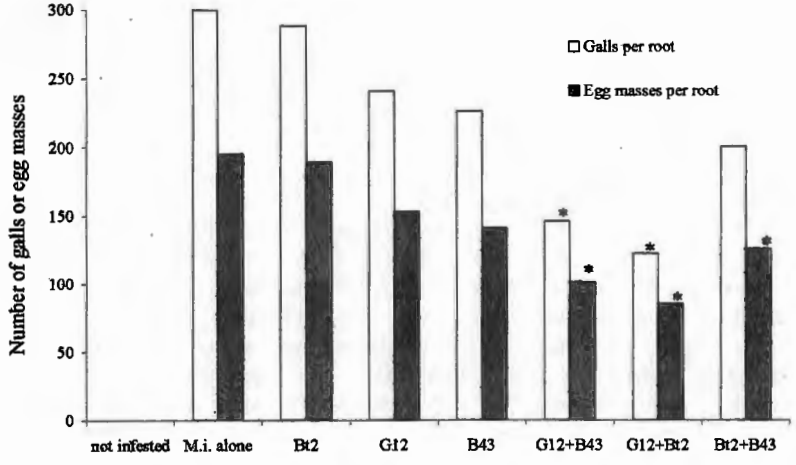

Figure 2. Numbers of *Meloidogyne incognita* galls and egg masses per root.
Three week old tomato plants (cv Hellfrucht Früher Stamm) were treated with rhizobacteria alone or in combinations as a soil drench. Overall bacteria densities were equal in all treatments. 2700 *M. incognita* eggs were inoculated three days later. Galls and egg masses were counted after 7 weeks. Treatments: not infested: no bacteria, no nematodes; M.i. alone: no bacteria treatment; Bt 2: *Bacillus thuringiensis* 2; G12: *Rhizobium etli* G12; B43: *Bacillus sphaericus* B43. Values with statistically significant differences (Dunnett T-test, $p \leq 0,05$; $n = 5$) compared to untreated infested plants are marked with an asterisk *.

Mode of action of effective bacteria strains

We are interested in the mode of action of efficient rhizobacteria. Better understanding of interactions between the host plant, the pathogen and the biocontrol agent should lead to optimizations in the application of biological control. We therefore conducted a series of experiments with the aim to characterize the mechanisms involved in biocontrol of *F. oxysporum* and *M. incognita* by rhizobacteria. Direct interactions between effective rhizobacteria and *M. incognita* or *F. oxysporum* were assessed in different *in vitro* systems. No significant effect was observed for any of the combinations tested. Plant mediated effects were studied in different set-ups with the aim to separate the inducer from the pathogen. Split root experiments were the method of choice for nematode experiments, whereas shoot cuttings were appropriate for tests on *F. oxysporum* symptoms. Results are summarized in Table 2. The two *Pseudomonas* strains induce resistance against *F. oxysporum* (Mwangi et al., in preparation). *B. thuringiensis* 2 and *R. etli* G12 induce resistance against *M. incognita* and *Bacillus sphaericus* B43 is able to induce resistance against both (Mwangi et al., in

preparation). Hasky-Günther et al. (1998) already showed that *R. etli* G12 and *B. sphaericus* B43 induce resistance against cyst nematodes on potato. The mode of action of the *Pseudomonas* strains against nematodes remain to be elucidated. This is particularly interesting for *P. fluorescens* T58, a strain that is able to reduce infection of *M. incognita* on tomato and of *Heterodera schachtii* on sugar beet.

Table 2. Specifity of resistance induction for the rhizobacteria tested in this study.

	Induction of systemic Resistance against	
Strain	*Meloidogyne incognita*	*Fusarium oxysporum* f.sp. *lycopersici*
Bacillus sphaericus B43	+	+
Bacillus thuringiensis 2	+	-
Pseudomonas fluorescens T58	not determined	+
Pseudomonas putida 53	not determined	+
Rhizobium etli G12	+	-

Mechanisms of induced resistance

We analysed changes in plant physiology after resistance induction by bacteria. Chlorophyll contents in the presence of *F. oxysporum* were higher after treatment with *B. sphaericus* B43 and *P. fluorescens* T58 when compared to *F. oxysporum*-infested or healthy plants. The same bacteria suppressed alterations in phenol compositions in the presence of *F. oxysporum*. Transient changes in activities of chitinases and different peroxidase isoforms were detected for *B. sphaericus* B43, *P. fluorescens* T58, and *P. putida* 53 (Table 3). However, timing of these enzymatic changes does not seem to correspond to defense against *Fusarium* infection and occured only in organs distant from the site of infection. These changes are probably not alone responsible for resistance against *Fusarium*. Electrophoretical separation of total proteins from roots revealed changes in protein composition, but the information from these experiments alone is not sufficient for a functional analysis.

Strategies for the characterization of the plant response

As biochemical analyses apparently did not reveal the decicive mechanisms in induced systemic resistance against plant-parasitic nematodes and soilborne fungi, we decided to analyse differences in protein patterns and in gene expression wih the aim of isolating genes that are differently regulated during induction of systemic resistance. Differential accumulation of proteins is being analysed after 2-dimensional Gel electrophoresis. A modified "Differential Display" technique is actually being used to select differentially expressed genes. Induced and untreated plants will be used as starting material for mRNA isolation and reverse transcription. Following specific amplification, PCR-products corresponding to differentially expressed genes are obtained. These will be used to isolate complete cDNA clones. Their sequences shall then give information on mechanisms of defense against nematodes or soilborne fungi. These "Rhizobacteria-induced genes" will be used for expression analyses under different conditions, and the function of their encoded

proteins will be characterized. Finally, we suppose that "Rhizobacteria-induced genes" may be a new tool for the control of soilborne pathogens and nematodes.

Table 3. Rhizobacteria-induced plant resposes related to induced systemic resistance. -: no effect; +: increase; ++: strong increase; n.d.: not determined

	Plant response mediated by rhizobacteria			
Strain	Increase in Chlorophyll content	Changes in phenol patterns	Induction of "new" proteins	Changes in defense-related enzyme activities
B. sphaericus B43	+	+	+	+
B. thuringiensis 2	n.d.	-	n.d.	n.d.
P. fluorescens T58	++	+	+	+
P. putida 53	-	-	+	+
R. etli G12	n.d.	-	+	-

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Mycofumigation with *Muscoder albus* for control of soil-borne microorganisms

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Abstract: Mycofumigation is the use of antimicrobial volatile chemicals produced by fungi such as *Muscoder albus* for the control of other microorganisms. Volatile organic compounds produced by this endophytic fungus include the following chemical classes: alcohols, esters, ketones, acids and lipids. The composition of the growth medium greatly influences the quantity and spectrum of compounds produced. The majority of the research done to date has involved growth on potato dextrose agar or growth on autoclaved barley. Volatiles produced on these media or a synthetic artificial mixture of component gases killed or inhibited a wide range of plant pathogenic fungi and bacteria in vitro and in vivo. Both glasshouse and field experiments show control of *Rhizoctonia solani, Pythium ultimum, Aphanomyces cochliodes, Verticillium dahliae* and *Streptomyces scabies*. In limited experiments these volatiles have shown no effect on the nematodes *Meloidogyne incognita and Heterodera schachtii.* Interesting is the fact that the biological control fungi in the genera *Trichoderma* and *Gliocladium* are unaffected by *M. albus*. *M. albus* is only one of several species identified to date that produce antibiotic volatile gases. M. albus and other Muscoder species have been licenced to AgraQuest Inc., Davis, CA. USA.

Key Words: mycofumigation, Muscoder species, biological control

Introduction

Muscoder species are recently discovered rainforest fungal endophytes that produce volatile organic compounds (VOC) that are antibiotic to a wide range of fungi and bacteria (Strobel et.al, 2001). The crucial observations implicating VOCs were made with endophytes collected from *Cinnamomum zeylanicum* in the Honduran rainforest, west of LaCeiba. After 7-10 days incubation at 24°C in a plastic box, only one fungus out of many that had started to grow from the plant material, remained alive in the plastic box. This fungus emitted gasses that killed virtually all other endophytic fungi that had been isolated from the stem tissue and placed in the box, even though each of the plates that had been originally placed in the box had been wrapped with parafilm. The gas producing fungus was named *Muscoder albus* (Worapong, 2001). Since the discovery of *M. albus*, a total of five gas producing fungi which appear to be very similar have been isolated. One strain, designated A3-5 and classified as *M. roseus* (Worapong, 2002), also exhibits excellent mycofumigant activity. Another isolate, which has been named *Muscodor vitigenus*, (Daisy et al., 2002a) was isolated as an endophyte from *Paullinia paullinoides* and produces naphthalene and has shown repellent activity against the wheat stem sawfly (Daisy, et al., 2002b.).

Although some fungal species have been known to produce low concentrations of volatile substances, to date, none have been demonstrated to produce antimicrobial volatiles (Bjurman and Kristensson, 1992). Some innocuous volatile substances may be common to many fungi, whereas others seem to be unique for one species (Schnürer, 1999). The lethal gas phenomenon that exists with Muscoder species represents a unique opportunity to develop

and test methodologies that may provide alternatives to soil applied pesticides in a wide range of applications.

Understanding the taxonomic position of *M. albus* is critical to evaluation this fungus as a biological control agent. After growing *M. albus* on at least ten different media, including the shavings of various woods, no fruiting structures have ever been observed. Genomic DNA was purified from "isolate 620" mycelium and the 18S rDNA gene was amplified by PCR and sequenced. "Isolate 620" was identified as being related to *Xylaria* species based on 97-98% homology to the sequence reported for several *Xylaria* sp. in the DNA data bank. In addition, its ITS1-2 sequences are related, but not identical to those of Xylaria sp. in the world's data bank (variation of 89-92% sequence homology). According to Bayman et al. (1997), *Xylaria* species are common ascomycetous endophytes found in tropical plants (Bayman et al., 1997). However, in this case, "isolate 620" cannot be named *Xylaria* sp. because no fruiting structures have ever been observed. Furthermore, *Xylaria* itself has never been noted to produce microbially lethal gasses (Dr. Jack Rogers of Washington State University, personal communication). Therefore, we have been left with the taxonomic alternative of naming "isolate 620" as *Muscodor albus,* a new fungal genus and species (Worapong et al., 2001). One point of interest is that the gasses produced by *M. albus* have no effect on non-gas producing members of the *Xylariales.* Due to differences in the profile of gasses produced, and also differences in culture characteristics, the isolate A3-5 has been named *Muscodor roseus* (Worapong et al., 2002). The absence of fruiting structures and their unique ecological niche, that of an endophtye, suggests that these fungi will not be soil colonists and that their host range will be highly limited. They grow well on several substrates and we believe that they could be introduced as mycofumigants on colonized substrates and die as the substrate is consumed. Alternatively, the critical chemical gas components have been identified and could be synthesized for use as a biorational fumigant.

In experiments not yet published, we have shown that *M. albus* is not a pathogen or phytotoxic to strawberry, sugar beet, eggplant, pepper, potato, tomato, chrysanthemum, barley, wheat, mint, tomato, spotted knapweed, chickpea, maize or canola. We have made multiple unsuccessful attempts to reisolate these fungi from several plant species growing in soil that had been infested with *M. albus* in the form of infested barley grain. Also, in survival experiments, we have not been able to recover *M. albus* after being buried in field soil over the winter. In formulation storage experiments we have shown that the fungus is not recoverable from colonized substrates after 6-8 weeks if stored at room temperature, while storage at 4^0 and -10^0C allows 100% recovery at up to 14 months.

This paper will discuss in vitro spectrum of activity results, chemical composition and biological activity of various chemical classes of *M. albus* antibiotic gases, in vivo results in glasshouse and field experiments using sugar beet, potato and eggplant pathosystems.

Materials and methods

In vitro testing

The effect of *M. albus* mycofumigant gasses on various microorganisms was tested by removing a 2 cm strip from the mid section of a 10 cm petri plant containing potato dextrose agar (PDA) leaving two half moons of agar on either side of the plate. A 5mm plug of M. albus is placed on one half of the plate and a 5 mm plug of test fungus or a streak of bacteria is placed on the other half. Plates are then sealed with parafilm and incubated for 7 days with growth measurements being taken every 2-3 days. After 7 days, the test organism is moved (via agar bits, or restreaked) on to an agar medium in the absence of the gas producing microbe in order to determine if it is dead or has remained alive and only inhibited after

exposure to *M. albus*. We have also done these same experiments using the "sandwich plate assay where the test pathogen is grown on one plate and the fumigant fungus is grown on another plate; both lids are removed and the plates are sandwiched together and sealed with parafilm. This is a change from the split/plate assay should eliminate the possibility that any diffusible compounds could influence the results.

Characterization of gasses

Analysis of the gases produced by *M. albus* was performed in parafilm wrapped Petri plates of *M. albus* growing on PDA. Analysis of the volatile compounds produced by *M.albus* was carried out using a gas chromatograph (GC) (Hewlett Packard 5890 Series II Plus) and a mass-selective detector (MS). The column was a 30 m × 0.25 mm I.D. ZB Wax capillary column with a film thickness of 0.50μ. The carrier gas was Helium Ultra High Purity (local distributor) and the initial column head pressure was 50 kPa. The He pressure was ramped with the temperature ramp of the oven to maintain a constant carrier gas flow velocity during the course of the separation. A "Solid Phase Micro Extraction" syringe was used for the injection. The fiber material (Supleco) was 50/30 divinylbenzene/carburen on polydimethylsiloxane on a stable flex fiber. The fiber was exposed to the vapor phase above the sample in the sealed container for a period of 45 minutes. This was accomplished by drilling a small hole into the side of the Petri plate and then inserting the microfiber through the hole. The trapped sample was injected into the GC, leaving a film in the hot injection port for 30 seconds to adsorb the volatiles from the film. The GC was interfaced to a VG 70E-HF double focusing magnetic mass spectrometer operating at a mass resolution of 1500. The MS was scanned at a rate of 0.50 sec. per mass decade over a mass range of 35-360 amu. Data acquisition and data processing was performed on the VG SIOS/OPUS interface and software package. Initial identification of the compounds produced by *M. albus* was made through library comparison using the NIST database.

Testing of artificial mixtures of gas components in vitro

The classes of compounds listed in Table 2 include esters, alcohols, ketones, acids and lipids. Compounds within each class were combined and tested for biological activity in plate assays. The amount used was based on the proportions of the different volatiles that were measured as they occurred in the gas phase above *M.albus* cultures. The test mixture was placed in a presterilized microcup (4x6 mm) located in the center of a Petri plate containing PDA. When not in use, the mixture was stored at 0°C. The test organisms, freshly growing and excised on $3mm^3$ agar blocks (at least 3 agar blocks per test fungus), were placed 2-3 cm from a microcup that had been filled with test mixtures of the volatiles. The plate was wrapped with two layers of parafilm. Measurements were made on mycelial growth from the edge of the agar blocks after 2 days.

In vivo testing

A bioassay for testing the ability of the mycofumigant to kill pathogens in infested soil was developed using the sugarbeet and eggplant pathosystems as models. Autoclaved soil was separately infested with the fungi *Rhizoctonia solani, Pythium ultimum,* and *Aphanomyces cochlioides. Rhizoctonia solani* AG 2.2 and *Verticillium dahliae*-infested soil was prepared by adding 5g of infested ground barley (mycelia and sclerotia) to each kg of autoclaved soil mix (equal parts soil, peat and sand). *Pythium ultimum* and *A. cochlioides*-infested soil was prepared by homogenizing one completely covered 10 cm petri plate and mixing this with 6.4 kg of soil mix. In each case 375g of infested soil was added to a 10cm square pot and 2 g of *M.albus* formulation was added followed by another 50g of infested soil. An additional

mycofumigant treatment was also included where four 0.5cm x8.0 cmx0.5 cm strips of *M. albus* colonized agar were placed in the top layer of soil. Pots were then placed in plastic bags, 100 ml of water was added, and the bags were sealed. Pots were incubated for 7 days in the dark at room temperature. After incubation, pots were removed from the plastic bags and 25 untreated sugar beet (cv. Beta 8754) seeds were planted per pot at a depth of 1cm. Pots were placed in the glass house at 23+/-1^{0}C/18+/-1^{0}C (day/night, 16 hr. photoperiod) and percent seedling survival was observed for 3 weeks. *Aphanomyces cochlioides* infested pots were placed in standing water in the greenhouse for one week after planting. For eggplant assays 3 week old seedlings (cv. Black Beauty) were planted into the test pots and maintained in the glasshouse under the same conditions described above. Disease severity was evaluated after 28 and 35 days. Four different formulations of the mycofumigant were prepared using 7-day-old cultures of *M. albus* and included pesta (Connick et al., 1991), alginate (Walker and Connick, 1983), stabileze (Quimby, et al., 1999), and colonized ground barley.

Assays testing a synthetic cocktail of the volatile compounds produced by *M. albus* for control of *Rhizoctonia solani, Aphanomyces cochloides,* and *Pythium ultimum* were done as follows. Treatments included an untreated control, *Rhizoctonia* control, *M. albus, M. roseus,* and a cocktail of the volatiles. Ten g. of autoclaved sieved soil was infested with pathogens as previously described and 60µl of cocktail was added to a mini-cup which was placed on the surface of the soil. Mycofumigation was done on in parafilm sealed petri plates at 23+/-1^{0}C for 1 week. An assay for damping off was then conducted by filling 10cm square pots with 400cc sieved, autoclaved soil mix. The soil from the fumigation treatments was layered on top of the sterile soil, 25 sugarbeet seeds were placed on the surface and seeds were covered with 80 cc of autoclaved soil. Pots were watered carefully and placed on greenhouse bench and after 2 weeks percent seedling establishment was noted

Potato field experiments were done in Ronan, MT in a field with severe *R. solani* AG-3 and *Streptomyces scabies* infestation using the potato cultivar, Ranger. *M. albus* (ground barley fomulation) was used as a seed treatment at 33 kg/ha. This treatment was compared to PCNB and azoxystrobin applied in-furrow. The experimental design was a complete randomized block. Potato plots consisted of 6 m rows with a 0.3 m plant spacing with 0.8 m row spacing. Rhizoctonia inoculum was applied in-furrow with the potato seed pieces. Treatments included three rates of PCNB, one rate of azoxystrobin alone and in combination with the high rate of PCNB. All chemical treatments were applied as a spray in-furrow. Rhizoctonia stolon canker ratings were performed on August 20 and scab ratings were done at harvest.

To determine the potential for mycofumigation under plastic mulch in the field, an experiment was set up in a Bozeman silt loam where packets of sugar beet pathogen infested soil used in glasshouse experiments was mycofumigated in the field then brought back to the greenhouse where seedling establishment assays were performed. Four nylon tubes containing 200g of soil each infested separately with the pathogens *Aphanomyces cochlioides, Pythium ultimum, and Rhizoctonia solani* were use for each replicate were attached to a string at 10 cm intervals. Five replications of the three pathogen treatments and an untreated control were buried in the soil so that the four samples were arranged in the soil profile at 0, 10, 20 and 30 cm. *M. albus* formulated in ground barley was applied to the surface of the soil using a fertilizer spreader at a rate of 480kg/ treated ha. Black plastic mulch and drip tape were then applied with a mechanical bedder mulch layer which disked approximately 10cm of loose soil over the surface where the mycofumigant was applied. Drip irrigation was applied 1 day after bed preparation. After one week, the plastic mulch was removed and the treated soil samples were dug out of the soil and taken back to the greenhouse. 25 sugar beet seeds (cv. HH88)

were planted into pots with the treated soil and seedling establishment was recorded after 2 weeks.

Results and Discussion

In vitro assay

Results of in vitro plate assays are found in Table 1.

Table 1. Influence of the lethal gasses of *M. albus* on several plant pathogens.

Fungus or Bacterium being tested in the plate assay	Growth in presence of *M. albus* after 2 days? (Y/N)	Growth when transferred to a new plate after 2 days? (Y/N)
Aspergillus fumigatus	N	N
Candida albicans	N	N
Cercospora beticola	Y	Y
Drechslera teres	N	N
Fusarium solani	N	Y
Geotrichim candidum	N	Y
Penicillium roqueforti	N	N
Phytophthora cactorum	N	N
Phytophthora cinnemoni	N	N
Pythium sp. (several)	N	N
Rhizoctonia solani	N	N
Sclerotinia sclerotiorum	N	N
Verticillium dahliae	N	N
Fusarium oxysporum f.sp. betae	N	Y
Fusarium sambucinum	N	Y
Helminthosporium solani	N	N
Erwinia carotovora	N	N
Erwinia amylovora	N	N
Escherichia coli	N	N

Gas chromatograph and mass spectrum analysis of gasses produced by *M. albus* are given in Table 2.

The classes of compounds listed in Table 2 include esters, alcohols, ketones, acids and lipids. . The esters, followed by the alcohols, had the greatest amount of inhibitory activity against the test fungi. The ketones, acids and lipids only caused a slight reduction in the growth of some fungi and none in others (Table 3.)

Table 2. GC/MS analysis of the volatile compounds produced by *M. albus.* Several minor peaks and the breakthrough peak were omitted from the total analysis since they represent only 1% of the total area. Compounds found in the control PDA plate are not included in this table.

RT	Total Area (%)	M/z	Possible compound	MW
3:45	0.33	114	Octane	114
4:19	0.93	58	Acetone	58
4:37	0.68	74	Methyl acetate	74
5:56	7.63	88	Ethyl acetate	88
6:51	0.31	102	Propanoic acid, 2-methyl, methyl ester	102
7:16	6.24	*	Ethanol	46
8:03	2.07	116	Propanoic acid, 2-methyl-ethyl ester	116
8:17	2.2	*	Acetic acid, 2-methylpropyl ester	116
11:45	0.58	*	Propanoic acid, 2-methyl 2-methylpropyl ester	144
12:05	2.06	74	Isobutyl alcohol	74
12:50	22.24	*	1-butanol, 3-methyl, acetate	130
14:57	1.53	*	Propanoic acid, 2-methyl, 3-methylbutyl ester	158
15:28	22.99	*	1-butanol, 3-methyl-	88
16:08	0.29	138	#Furan, 2-pentyl-	138
18:53	0.29	142	#4-nonanone	142
20:38	0.41	142	2-nonanone	142
21:07	0.30	204	# Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR-trans)-	204
22:54	1.51	204	# Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,[1S-(1.alpha.,4.alpha.,7.alpha.)]	204
23:16	0.94	204	# Cyclohexene, 4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-	204
25:20	3.63	204	# 1H-3a,7-methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8 tetramethyl-, [3R-(3.alpha., 3a.beta.,7.beta.,8a.alpha.)]	204
25:30	6.08	88	Propanoic acid, 2-methyl	88
26:04	0.48	204	Caryophyllene	204
27:55	0.34	204	# Naphthalene,1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1R-(1.alpha., 4a.alpha.,8a.alpha.)]	204
28:34	0.36	204	# Spiro[5.5]undec-2-ene,3,7,7-trimethyl-11-methylene-	204
28:50	1.07	204	Azulene, 1,2,3,5,6,7,8, 8a-octahydro-1, 4-dimethyl-7- (1-methylethyenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)] Common Name: Bulnesene	204
28:57	3.24	204	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-,[1R-(1.alpha.,7.beta.,8a.alpha.)] Common Name: Valencene	204
31:12	1.74	*	Acetic acid,2-phenylethyl ester	164
33:17	1.06	122	Phenylethyl alcohol	122
39:00	9.76	204	# Unknown	204

* No molecular-ion peak was observed in the spectrum of either the standard compound or the compound undergoing the analysis. # Denotes that a spectrum and retention time of this component was observed and the substance matched to the most likely compound in the NIST data base, but the data have not been confirmed by use of an appropriate identical standard compound by either retention time or MS. These compounds were not placed in the artifical mixture in the bioassay test.

Results of formulation trials are presented in Table 4. Stand establishment with the *M. albus* stabileze and barley formulations was significantly better than the inoculated control for all pathogens tested. The barley and stabileze formulations produced results similar to the untreated control for *Rhizoctonia* and the stabileze formulation was similar to the untreated control in the *Pythium* experiment.

Table 3. The inhibitory influence of each class of volatile compounds is expressed as the percent of the test fungal growth as compared to a control not in the presence of the test compounds. The compounds were tested for a 2 day exposure at the relative concentrations that they occur in *M. albus* at the optimum test concentration 60µl/50 CC air space or 1.2µl /CC.*[1]

Test fungus [1]	Alcohols 0.48 µl/CC % growth of control	Esters 0.53 µl/CC % growth of control	Ketones 0.02 µl/CC % growth of control	Acids 0.09 µl/CC % growth of control	Lipids 0.08µl/CC % growth of control
Pythium ultimum	11.2 ± 4	0	67.5 ± 7	40.9 ± 3	75 ± 0
Rhizoctonia solani	55± 5	0	67.5±7.5	67.5±7.5	40±0
Tapesia yallundae	35±15	0	75 ± 25	100± 0	100±0
Xylaria sp.	75±25	0	100±0	100±0	100±0
Sclerotinia sclerotiorum	29±3	8.1±1.5	20.6±12	40±0	78±2
Cercospora beticola	58±8	5 ± 5	100±0	83±17	100±0
Fusarium solani	70±10	55± 5	90±10	80±20	80±10

*All measurements of mycelial growth compared to the control were made as described in Table 1.

[1]None of the microbes was killed after a three day exposure to any of the artificial test mixtures given on this table.

While the efficacy of the *M. albus* was not as dramatic as for the other two pathogens, it was still very good considering the rigor of the assay conditions where the plants were left in standing water for 2 weeks (Table 4.). These experiments have been repeated multiple times with similar results. We have also repeated these experiments without bagging the pots and attained similar results. Containment of the gases does not seem to be critical for mycofumigation efficacy.

Table 4. Percent seedling establishment of sugarbeet two weeks after planting into *Rhizoctonia solani* AG 2-2, *Pythium ultimum, or Aphanomyces cochliodes* infested soil that had been mycofumigated with different formulations of *Muscodor albus*

	Pathogen Used to Infest Soil		
Treatment	***Rhizoctonia***	***Pythium***	***Aphanomyces***
Uninoculated Control	88a[1]	77a	85a
Pathogen Control	38d	8cd	20c
M. albus Agar	82ab	22c	
M. albus Pesta	57c	5d	
M. albus Alginate	65bc		
M. albus Barley	83a	58b	54b
M. albus Stabileze	80ab	67ab	34c
$LSD_{(0.05)}$	17.2	17.1	16.7

[1]Means followed by the same number are not significantly different.

Mycofumigation with the stabileze formulation of *M. albus* and *M. roseus* was done on soil infested with *V. dahliae* 7 days. In one experiment soil dilution plating revealed no detectable

V. dahliae in the infested mycofumigated soils, 8.1 x 10^5 cfu/g soil in the *V. dahliae* infested, non-mycofumigated soil and none in the non infested, non mycofumigated soil. Data from two other experiments are presented in Table 5. Visible wilting of eggplants was present in the *V. dahliae* treated soils within 3 weeks while most plants transplanted into mycofumigated soil escape infection. Some plants in the mycofumigated soil became diseased but the latent period between transplanting and disease development was increased 1 to 2 weeks and overall symptoms were less severe. M. albus mycofumigation reduced both symptom development as measured by disease index and cfu of *V. dahliae*.

Table 5. *Verticillium dahliae* soil population (cfu/g of dry soil) after infested soil was mycofumigated for 7 days by *M. albus* using the stabileze formulation compared to non-mycofumigated soil. Initial population of *V. dahliae* was 1.64 x10^4cfu/g of dry soil.

Treatment	(cfu/g)	DI[2] (4 weeks)	DI (5 weeks)
Autoclaved , non-infested control	0 b[1]	0.0b	0.0c
V. dahliae	66840 a	55.0a	60.0a
V. dahliae +*M.albus*	1257 b	10.0b	20.0bc

[1]Means followed by the same letter are not significantly different at $p< 0.05$. Each value is the mean of 15 replications from 2 experiments. [2]Disease Index was based on a scale of 0-3, with 0=symptoms absent, 1= symptoms slight, may have small areas of chlorosis, transplant not stunted 2= symptoms moderate, leaves yellowing, partial wilt of a couple of leaves, slightly stunted 3=complete wilt, all leaves affected and transplant severely stunted. Disease index was calculated using the following calculation:

DI=$\frac{\Sigma(\text{Number of plants in each severity class x class number})}{\text{(Mean number of plants grown in autoclaved soil x number of disease classes)}}$ X 100

Data on disease control assays using a cocktail of the volatile compounds produced by *M.albus* for control of *Rhizoctonia solani, Aphanomyces cochloides,* and *Pythium ultimum* are found in Table 6 . Two weeks after planting, emergence of sugarbeet planted in the *M. albus, M. roseus*, and the uninoculated control soil were similar and significantly higher than the all of the pathogen inoculated controls. Cocktail treated soil was similar to the untreated control for the *Rhizoctonia* and *Pythium* treatments, but fell between the uninoculated and the inoculated control treatments for the *Aphanomyces* (Table 6).

Table 6. Percent seedling establishment of sugarbeet two weeks after planting into *Rhizoctonia solani* Ag 2-2, *Pythium ultimum*, and *Aphanomyces cochliodes* infested soil that had been mycofumigated with *Muscodor albus*, *Muscodor roseus*, or a "cocktail" of volatile compounds based on the profile of compounds produced by *M. albus*.

Treatment	Percent Seedling Establishment		
	Rhizoctonia	*Pythium*	*Aphanomyces*
Uninoculated control	99a[1]	98a	90a
Pathogen inoculated control	17b	1b	1c
Muscodor albus	92a	86a	89a
Muscodor roseus	95a	86a	90a
Cocktail	92a	99a	61b

[1]Means followed by the same number are not significantly different.

Data on effect of mycofumigation in the field are found in Table 7. When seedling establishment was averaged over all depths, the field mycofumigated *Aphanomyces* and *Pythium* treatments both had seedling establishment significantly higher than the pathogen inoculated controls, but lower than the uninoculated controls. The *Rhizoctonia* inoculum was too high and seedling emergence was not improved by mycofumigation Seedling establishment for mycofumigated treatments was best at 10cm for *Aphanomyces* and 0cm for *Pythium*.

Table 7. Sugar beet seedling establishment in pathogen infested field soil, buried from 0 to 30cm, then mycofumigated with *Muscodor albus* for 1 week

	Percent Seedling Establishment at Four Depths				
Treatment	All Depths	0 cm	10 cm	20 cm	30 cm
UTC	81a	79a	77ab	82a	87a
UTC + MF	78a	43bc	86a	91a	91a
Aphanomyces	26cd	64ab	29c	8c	5c
Aphanomyces + MF	46b	44bc	60b	36b	44b
Pythium	16de	24cd	20c	14c	15bc
Pythium + MF	33bc	45bc	11c	34b	34bc
Rhizoctonia	13de	9d	10c	11c	21bc
Rhizoctonia + MF	8e	3d	18c	7c	6c

[1]Means followed by the same number are not significantly different.

Data on effect of *M. albus* seed treatment and in furrow application of PCNB and azoxystrobin are presented in Table 8. *M. albus* treated plots had equivalent disease and yield as the PCNB + azoxystrobin treatment. There was a slight reduction in disease by *M. albus*, but not significantly less than the untreated control. There was not enough black scurf to perform ratings on the tubers. Scab ratings performed after harvest showed equivalent scab ratings for the *M. albus* and all of the chemical treatments except the highest rate of PCNB. The *M. albus* treatment was lower than the untreated plots, but not quite enough to be

significant considering the variability of the infection in the plots (Table 8). While these results do not show a high level of significance in the decrease in the two diseases due to mycofumigation, there is a trend towards disease reduction similar to what is seen with the optimal chemistry. This experiment was performed with only one rate of *M. albus* and based on our work with the sugarbeet pathosystem, we expect a greater response in disease control would be achieved with a higher rate of the mycofumigant.

Table 7. Effect of in-furrow application of *Muscodor albus* on two potato disease compared to chemical standards

Treatment	Rhizoctonia stolon canker Percent Disease	Streptomyces Scab Percent Disease
Untreated Control	14	22
Muscodor albus	10	17
PCNB – 5.8 l/ha	18	14
PCNB – 8.7 l/ha	18	16
PCNB – 11.6 l/ha	7	11
Azoxystrobin 1.45ml/100m	4	15
Azoxystrobin 1.45ml/100m + PCNB – 11.6 l/ha	10	16
$LSD_{(0.05)}$	8.1	6.4

Discussion

Mycofumigation with *M. albus* was effective in reducing disease severity associated with the soil borne plant pathogens: *R. solani, P. ultimum, A. cochlioides* and *V. dahliae*. The percent stand establishment of sugar beet seedlings in treatments using *M. albus* was consistently greater, and the disease index was lower relative to the non-mycofumigated pathogen infested soil. In similar experiments comparing *M. roseus* and *M. albus*, *M. albus* was more effective on all pathogens except *P. ultimum* (Stinson et.al, 2003). Neither *M. roseus* nor *M. albus* reduced soil populations of *F. oxysporum* f.sp. *betae* or disease severity caused by this fungus (Stinson et.al, 2003). This was consistent with *in vitro* tests, which showed only inhibitory but non-lethal effects of the mycofumigant gas on *F. oxysporum* f.sp. *betae* (Strobel et.al, 2001). Our results indicate that both *M. roseus* and *M. albus* reduce disease severity caused by *Verticillium dahliae* ((Stinson et.al, 2003). *M. albus* mycofumigation significantly reduced population levels of *V.dahliae*. Because only six to fifty microsclerotia per gram is sufficient to give 100 percent infection in most susceptible crops (Agrios, 1997), this level of control is not acceptable. However, we hypothesize that if our initial inoculum had been more in the range of 10-50 cfu/g, a parallel amount of reduction in inoculum level could result in undetectable levels of the pathogen. Experiments focusing on the mechanism of population reduction and on the effect of inoculum level need to be carried out before it can be speculated as to the efficacy of *M. albus* mycofumigation in the field.

Mycofumigation has shown potential for control of a wide range of soil-borne pathogens and for management of both seed infecting pathogens (Ezra and Strobel, 2003). The mycofumigant fungi tested to date have shown no phytotoxicity to plants and experience has shown that they die out when their substrate is depleted. Future research should focus on development of optimal formulations and a better understanding of the most important antimicrobial VOC produced by these fungi. Recent research by Ezra and Strobel (2003 has

shown that the VOC produced by *M. albus* vary with the growth medium used. Ground barley based mycofumigant has consistently been a highly effective formulation for both field and glasshouse use.

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Effect of soil nutrients on the growth, survival and fecundity of insect pests of rice: an overview and a theory of pest outbreaks with consideration of research approaches

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Abstract: The addition of nutrients to the soil, especially nitrogen, is known to increase the growth, survival and fecundity of many rice insect pests. Likewise, an environmental stress, such as drought, which increases the availability of soluble nitrogen in the plant could be expected to have similar effects on the insects' biology. An increase in pest populations normally leads to an increase in the immigration and reproductive rates of natural enemies so that pest outbreaks are prevented. If the population growth response of natural enemies is prevented or sufficiently delayed, however, then pest outbreaks may occur. Such interference in the response of natural enemies could result from insecticide use or from environmental conditions that permit predators of the natural enemies to enter the agroecosystem. This paper contains an overview of the literature on the relation between soil nutrients and insect pest outbreaks on rice, a proposal that the rice mealybug outbreaks of South Asia are the result of drought induced changes in plant metabolism and ant-mealybugs mutualism, and a review of possible research approaches that can be used to understand how multitrophic interactions and nutritional effects can contribute to the occurrence of rice pest outbreaks.

Key words: rice, insect pests, fertilizer

Introduction

This paper briefly presents what is know and not know about how nutrients effect the growth, fecundity and survival of insect pests of rice. This is followed by a proposed theory on how the effects of available nitrogen on insect reproduction might be related to pest outbreaks when multitrophic interactions are disturbed, using the rice mealybug outbreaks as an example. Finally, several possible research approaches to understanding how plant nutrition is related to pest outbreaks are presented.

Some types of fertilizer application to rice, particularly of N, tend to increase pest numbers, survival, fecundity, body weight, and damage (Heinrichs 1994). Do fertilizer applications increase pest levels overall? And do such pest increases result in greater crop loss? Do natural enemy populations respond to fertilizer-induced increases in pest populations? Is the natural enemy population response rapid enough to prevent yield loss resulting from insect damage? Would the effect be the same on calcareous soils as on other soil types? Currently, there is simply not enough information available to allow us to predict how changes in rice cultivar and fertilizer regimes will affect pest problems on different soil types at different locations. If such predictions were possible, then in theory some pest problems could be prevented or controlled by manipulating those interactions.

Overview of literature

There has been a steady shift in rice production, since the late 1960s, toward semidwarf, nitrogen-responsive varieties. This change is believed by some to have caused increased pest problems (Nickel 1973, Mew 1992), though the role of fertilizer has not been clearly established or well understood. There is no simple formula to describe how plant recovery from herbivory is related to the availability of soil nutrients. The "continuum of responses" model (Maschinski and Whitham 1989), for example, predicts that plants are best able to recover from pest damage when well fertilized, while the "growth rate" model (Hilbert et al 1981) predicts that damaged plants will exhibit superior recovery from herbivory when grown under stress, i.e. low levels of nutrients. Meta-analysis suggests that basal meristem monocots, such as rice and other grasses, exhibit better recovery from pest damage when the plants are grown under high resource levels; while dicots show better recovery when grown under low resource levels (Hawkes and Sullivan 2001).

Excess fertilizer can lead to increased pest levels (Litsinger 1994). This is, of course, an oversimplification of the problem. How fertilizer applications affect the severity of pest damage will depend not only on the amount of fertilizer but also on the composition and timing of the applications. The proper balance of nutrients can help keep pest incidence low. Studies in India, China, Indonesia, the Philippines, and Vietnam have found lower pest incidence in fields with site-specific nutrient management compared with the farmers' fertilizer practices (Sta. Cruz et al 2001). N applications apparently decrease thrips populations in rice fields (Ghose et al 1960). Other pests become more abundant if N is applied, such as weeds, sheath blight, leafhoppers, planthoppers and gall midge (Chelliah and Subramanian 1972, Oya and Suzuki 1971, Reissig et al 1985, Savary et al 1995, 2000a,b). Several species of stem borer larvae exhibit significant weight gains when N is applied to the host plant (Ishii and Hirano 1958, Rubia 1994,). Heavier stem borer larvae presumably cause more damage to the host plant than lighter larvae. More stem borer (*Chilo suppressalis* (Walker)) eggs are found in fields with high N rates (Hirano 1964); and stemborer larval survival is highest on plants receiving N (Alinia et al. 2000).

In general, what is good for the plant is good for the pest, and this is particularly true of N applications (Litsinger 1994). This is why most pest populations will increase along with rice yields, that is, pest populations are often positively correlated with rice yields. Although there are exceptions among rice-feeding insects, N applications tend to promote greater survival, increased tolerance of stress, higher fecundity, increased feeding rates, and higher populations (Uthamasamy et al 1983). N applications also make rice more attractive to many herbivorous insects (Mattson 1980, Maischner 1995). N augments rice plant growth, resulting in softer plant tissues, which presumably allows for easier penetration of the rice plant by insects and pathogens (Nadarajan and Janardhanan Pillai 1985, Oya and Suzuki 1971). But why then do N applications tend to decrease thrips populations? High N rates generally attract ovipositing insects and increase insect fecundity, though it is not known why. Other nutrients, such as P improve root development and tolerance for root pests (e.g., the root weevil (*Echinocnemus oryzae* Marshall) (Tirumala Rao 1952). How NPK interactions affect root pests, however, is not understood. Unlike N applications, K applications tend to suppress pests by lowering plant sugar and amino acid levels, promoting thicker cell walls, and increasing silicon uptake (Baskaran 1985).

Minor plant nutrients can also contribute to pest suppression. In the proper quantities, silicon and zinc can increase the resistance of rice plants to blast, brown spot, bacterial blight, planthoppers, and stem borers (Chang et al 2001, Kim and Heinrichs 1982, Pathak et al 1971, Prakash 1999, Reddy 1967).

The effect of fertilizer on natural enemies has not been studied much in rice. Research from other crops suggests that some types of parasitoids concentrate their attacks on insect hosts that feed on leaves with the highest N content (e.g., Loader and Damman 1991).

A theory on the cause of rice mealybug outbreaks

A number of sporadic rice pests are known to cause severe crop loss in rainfed rice during or immediately following droughts. Rice mealybugs, *Brevennia rehi* (Lindinger), provide a particulary interesting example because they only achieve pest status during droughts (Alam and Karim 1981, Mammem 1976). Drought conditions upset the normal metabolism of the plant, making amino acids and soluble nitrogen more readily available to phytopagous insects (Fennah 1960), which increases the growth and fecundity of phytophages. However, under normal circumstances, an increase in the population of a phytophage is accompanied by an increase in the population of its natural enemies which prevents a pest outbreak. Ants are well known to tend mealybugs and protect them from natural enemies in pineapple and other crops (Jahn and Beardsley 1998, Jahn et al. in press). It seems reasonable that ants would play a similar role in the rice field. Under normal, flooded conditions ants are prevented from tending mealybugs in rice fields. When the field dries out ants could enter the field and protect mealybugs from parasitoids and predators. It is also possible that pathogenic fungi regulate mealybugs under normal conditions but not during droughts. Understanding the degree to which each of these factors contributes to particular pest problems in drought prone areas could well lead to improved nutrient management, cropping practices, and varietal selection that would reduce or prevent crop loss. The standard practice of applying insecticides to quickly control pests in water-stressed fields may not be the ideal solution.

Options for research approaches

Understanding how multitrophic interactions and soil nutrient effects in combination with environmental stress can lead to pest outbreaks in rice, could be approached deductively (Sta. Cruz et al 2001, Ludwig and Reynolds 1988), inductively (Platt 1964), or through a participatory approach known as action research (Checkland 1985, Gu and Zhu 1995, Röling and Wagemakers 1998). Each of these approaches has advantages (Table 1), suggesting that it may be ideal to combine the three approaches.

Table 1. Relative advantages of different research approaches, where 1 indicates the greatest advantage and 3 indicates the lowest advantage.

Approach	Deductive	Inductive	Adaptive
Multiple components	1	3	2
Predictive	1	2	3
Reveals mechanism	2	1	3
Increase understanding	2	1	3
Rapid application	3	2	1
Rapid adoption	2	3	1

Deductive Approach

Typically, deduction is used for modeling complex systems. It consists of describing the general situation and then (based on patterns, associations, or correlations) deducing the

expected outcome of specific interactions. By characterizing multiple components of a system, modeling can highlight which areas are poorly understood, and which components dominate the outcome (Norton et al 1991). This is not the same thing as prioritizing research, however, which requires drawing boundaries and deciding which components are more likely than others to contribute to the desired objective. In fact, thorough characterization may actually be an impediment to producing practical outputs. In an effort to make a model more predictive, it is tempting to continually add components until the model becomes so complex that it can never be applied in a real-world situation (Cox 1996).

The predictive nature of deduction should not be confused with determining causation. It is quite possible to correctly ascertain the association of events without knowing the mechanism for it. It is also possible to describe spurious associations in the mistaken notion that they are somehow causally linked. For instance, studying the coincidence of certain historical events with the appearance of celestial bodies led to the development of astrology. A deductive approach might begin with the spatial delineation of the soil properties, rainfall, and rice pests at landscape or regional scales. Then the interactions of these factors could be characterized, through nonparametric techniques (e.g., correspondence analysis) (Jahn et al 2000a,b). The relative contribution of pests to variation in yield can be deduced using step-wise regression techniques. Predictions of the model must then be rigorously tested and the model modified based on the test results.

Inductive Approach

The inductive approach moves from specific discoveries to generalizations about the way nature works. This is the classic scientific method, aptly described by Platt (1964) as "strong inference." In this approach, the scientist seeks to simplify the system to the point that variation is controlled in all components except one. The disadvantage to this approach is that a highly simplified system may not reflect the reality of farmers' fields. Using an inductive approach, researchers might first identify suspected cases of pest problems dominating the production situation and then conduct experiments to show that the phenomena can be reproduced in repeatable ways. Unlike the deductive approach, the inductive approach has an aspect of research prioritization already built into the research process. Only experimentally repeatable results merit further investigation. The mechanisms for the observed phenomena are identified by eliminating alternative hypotheses through decisive experimentation. Mechanisms of pest outbreaks could range from chemical and physiological causes to behavioral or ecological causes, thus requiring interdisciplinary investigations. By revealing fundamental causes of outbreaks, it should be possible to predict outbreaks and make integrated pest and nutrient management recommendations. If these predictions and recommendations are incorrect in some cases, then investigators would return to studying causation in more detail.

Action Research, a Participatory Approach

Action research is a form of adaptive management characterized by a strong emphasis on participatory approaches (Flood 2000). Through this approach, neither characterization nor causation is sought (Checkland 1985). Rather, the emphasis is on creating case studies of situations in which farmers and scientists have worked together to solve practical problems (e.g. Jahn et al 1999). Each case study serves as a basis for the next intervention, creating learning cycles. While traditional science may use farmers as a source of information, the adaptive approach includes farmers and other stakeholders as collaborators and colleagues, so that adoption of existing technology and adaptation to the farm situation are relatively rapid (e.g. Cox et al 1999a,b). Modeling may form an important part of the adaptive approach, but

with the end user in mind. In the deductive approach of system dynamics, modeling is a means for researchers to describe the universe in sufficient detail to predict events (Lane 2000). In contrast, adaptive research models are tools that farmers use to support their decisions. An adaptive model, for instance, could include crop calendars that aid pest management decisions. Because action research fosters a high degree of farmer participation, it tends to have a relatively high impact on farmers' livelihoods. The very process of conducting the research with farmers tends to ensure the relevance of research results to farmers' problems. Unfortunately, the adaptive approach is so tailored to specific circumstances that replication and, therefore, verification are often quite difficult. When an adaptive approach solves a problem, it may not be clear why since the objectives and methods of adaptive research are constantly shifting, resulting in messy data. To the degree that farmers are involved in the research, the treatments and results become increasingly heterogeneous and difficult to analyze (Petch and Pleasant 1994), although the relevance and sustainability of those results are usually increased (Chambers and Jiggins 1987, Cox 1998). Another concern is the evaluation of new technology with farmers. Should resource-poor farmers be exposed to the risks of unproven technology so that they can evaluate it? Perhaps the adaptive approach is better suited for developing solutions than for evaluating new technology or discovering mechanisms. An adaptive approach to pest outbreak research could begin by identifying pest-related production problems with farmers. After finding out how farmers are dealing with the problem, scientists and farmers could discuss new ways to solve the problem and then test solutions together. The results are documented, applied to the next case study, and the cycle continues.

A participatory approach to solving pest problems related to multitrophic interactions and nutritional effects might begin with a meeting of farmers and scientists to identify their respective desires (Gu and Zu 1995). Scientists may be concerned with understanding the cause of pest outbreaks while farmers are more concerned with improving rice yields, production or grain quality. Once farmers understand how the goals of the scientists can help them reach their own goals, farmers and scientists can begin investigating conditions and setting research objectives together. Based on the results of their mutual research, farmers and scientists develop adaptive research models to aid crop management decisions. Discussions between farmers and scientist should result in a set of recommendations which are implemented and monitored. Farmers and scientists meet again to discuss the results and to understand how each party wishes to proceed.

Combining Approaches

Possibly we can apply the best of each approach toward a research agenda. After identifying cases of pest outbreaks apparently related to multitrophic interactions and soil nutrients, the mechanism of these outbreaks could be determined by controlled experiments, i.e. inductive means. Deductive models would then be used to predict where and when such problems are likely to occur. Interventions to prevent the problems could then be developed and tested on a case by case basis through farmer participatory action research.

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Multitrophic Interactions in Soil and Integrated Control
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Application of *Pochonia chlamydosporia* in the integrated control of root-knot nematodes on organically grown vegetable crops in Cuba

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Abstract: An isolate of the nematophagous fungus, *Pochonia chlamydosporia*, indigenous to Cuba was added to soil infested with root-knot nematodes (*Meloidogyne* spp.) to evaluate its potential as a biological control agent against these pests on vegetable crops. The fungus significantly reduced nematode infestations when used in combination with poor hosts for the nematode in the crop rotation. The fungus can be readily grown *in vitro* and a simple method is described for the production of chlamydospores, which are the favoured source of inoculum for application to soil. The method enables sufficient inoculum to be produced for small scale field trials but there is a need to improve the efficiency and scale up production methods.

Key words: biological control; *Pochonia chlamydosporia*; root-knot nematodes; organic vegetable production; urban agriculture.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are an important constraint to vegetable production in warm temperate and tropical soils and are widespread in Cuba. In the major cities on the island, urban agriculture is widely practised, especially for the production of vegetables, and employs 300,000 workers. The holdings are usually < 2 ha in area and are situated in each of the major residential areas. Crops are produced organically and intensively and provide direct access to fresh vegetables to local residents, primarily to improve the diet of the urban poor (Murphy, 1999).

Cuba has an infrastructure of > 200 small production plants that produce biological control agents for the use of local growers. However, because of problems with quality control in some production processes uptake of microbial agents has been limited. Rothamsted Research in the UK and the Centro Nacional de Sanidad Agropecuaria, Havana have collaborated for the past 5 years to develop a fungal agent, *Pochonia chlamydosporia* (syn: *Verticillium chlamydosporium*) as a biological control agent for root-knot nematodes and to establish methods to maintain inoculum quality.

A survey was made of nematode egg parasitic fungi in coffee plantations infested with root-knot nematodes, which were known to be widespread (Hidalgo-Diaz *et al.*, 2000). Under a perennial crop it was considered that natural enemies had a greater opportunity to increase to detectable levels than in soils where annual crops were grown (Stirling, 1991). *Meloidogyne incognita, M. arenaria, M. javanica* and *M. mayaguenis*, which are the major root-knot nematode pests worldwide accounting for about US $70 billion crop losses each year, were all identified (Hidalgo-Diaz *et al.*, 2000). Their eggs were infected with the most diverse community of *Pochonia* isolates so far encountered and included *P. chlamydosporia* var *chlamydosporia, P. chlamydosporia* var *catenulata and P. suchlasporium* (Hidalgo-Diaz et al., 2000). An isolate of *P. chlamydosporia* var *catenulata* was selected for further testing

on the basis of its ability to produce chlamydospores and to colonise the rhizosphere of vegetable crops and its virulence against root-knot nematode eggs (Hidalgo-Diaz *et al.*, 2000). This paper briefly presents some early data on the production methods and evaluation of the selected isolate for the biological control of root-knot nematodes on vegetable crops.

Mass production and application of inoculum

Chlamydospores have been used as the preferred propagule for application to soil because they do not require an additional energy source to establish the fungus in the rhizosphere of plants susceptible to root-knot nematodes. However, the yields of chlamydospores in liquid fermentation systems are poor and solid media must be used to obtain sufficient inoculum for treatments in the field. Large-scale, solid media production systems are expensive to build and run and commercial production that can be achieved in existing liquid fermenters is the favoured option (Powell, 1993). However, in many developing agricultural systems access to such fermentation systems may be difficult but small-scale solid fermentation systems can be established relatively cheaply using sterile solid media in kilogram quantities in autoclaveable bags. A number of biological control agents have been produced on solid media in this way (Jones *et al.*, 1993) but preventing contamination and maintaining the quality of inoculum can be a significant problem and requires the careful adherence to standard operating procedures.

A biphasic production process has been devised to produce fresh inoculum of *P. chlamydosporia* in quantities sufficient for small scale field trials. The liquour from boiled rice is used as a medium for producing fungal biomass, which is transferred aseptically to sterile rice grains to promote sporulation. Cracked rice grain is used as a cheap medium. Chlamydospores are washed from the rice using standard methods (De Leij and Kerry, 1991) and yields of 10^6 spores g^{-1} medium are typical. Methods for larger scale production using autoclaveable bags and the collection of spores from dried medium using the Mycoharvester® (CABI, UK) are currently being optimised and chlamydospore numbers of 10^7 g^{-1} medium have been obtained. As recommended application rates for the control of root-knot nematodes require 10^3 chlamydospores g-1 soil, then 10 g of medium are needed to treat one tonne of soil, which is approximately 250 Kg of medium per hectare. Unless further improvements in production efficiency can be obtained, then in row or patch treatments with the fungus will be necessary to make the current production systems practical.

Evaluation of a biomanagement strategy

It has been widely reported (Kerry, 1995) that *P. chlamydosporia* will not control root-knot nematodes on highly susceptible crops but may cause significant reductions in nematode infestations when used in combination with poor hosts in the crop rotation. This strategy has been developed (Atkins *et al.*, 2003) and tested in a grower's field in Cuba and is briefly described. Following a badly damaged beetroot crop the soil contained approximately 30 second-stage juveniles of *M. incognita* g^{-1} soil. The area was divided into eight 2 m^2 plots of which half were treated with 12 x 10^7 chlamydospores of an indigenous isolate of *P. chlamydosporia* var *catenulata*. Treated plots were selected at random and the chlamydospores were incorporated in soil to a depth of 15 cm immediately before beans, which are a poor host for *M. incognita*, were sown. This crop was followed by cabbages another poor host for the nematode before a fully susceptible tomato crop was planted. There was only one application of the fungus but all crops supported its growth in their rhizospheres; the tomato crop was planted 96 days after the application of the fungus. After each crop harvest, yields were measured and the numbers of second-stage juveniles in soil and the proportion of nematode eggs colonised by the fungus estimated (Table 1). As expected, the bean and cabbage crops significantly reduced ($P< 0.001$) the numbers of *M.*

incognita in soil in both fungus treated and untreated soil. Fewer nematodes were found in soil treated with the fungus after the bean crop ($P< 0.05$) and the tomato crop ($P< 0.001$).

Table 1. The effect of an application of *P. chlamydosporia* on the numbers of second-stage juveniles of *M. incognita* in soil after different crops in the crop rotation.

Treatment	Cropping sequence				
	Beans	Cabbage	Tomato	% Fungal+ infection	
	Numbers of juveniles $100g^{-1}$ soil			Egg masses	Eggs
Fungus application	100^{a}	50^{c}	50^{c}	68^{a}	69^{a}
Untreated	130^{b}	70^{c}	320^{d}	4^{b}	2^{b}
	Root gall index				
Fungus application	2.6^{a}	0.1^{b}	2.5^{a}		
Untreated	2.7^{a}	0.1^{b}	4.0^{c}		

+ Estimates based on egg masses collected at harvest of the tomato crop only.

Approximately 70% of the nematode egg masses and eggs produced on the roots of tomato plants were infected with the fungus compared to only 7% in untreated soil, which contained *Paecilomyces lilacinus* and some *Fusarium* spp. Clearly, the fungus remained active in soil throughout the cropping cycle and significantly reduced nematode densities. Although there were no yield benefits in any of the crops from the application of the isolate of *P. chlamydosporia* used, the root gall index was reduced from 4 to 2.5 in the tomato crop, and it is anticipated that subsequent crops would benefit from the reduced numbers of nematodes in soil. The combined used of poor nematode hosts grown in the crop rotation and the application of selected isolates of *P. chlamydosporia* may provide a strategy for the management of root-knot nematodes on vegetable crops and this is currently being tested in different sites in Cuba and Kenya.

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Variation in *Pochonia chlamydosporia* and its potential as a biological control agent for root-knot nematodes.

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Abstract: The generalist, facultative fungus, *Pochonia chlamydosporia*, appears to demonstrate host preferences at the sub-specific level. This affects the selection of isolates of this fungus as biological control agents for cyst and root-knot nematodes. Phylogenetic analysis of data generated by ERIC-PCR indicated that isolates of the fungus grouped more strongly on the basis of their original host rather than by their geographic origin. Isolates collected from cyst nematodes were distinct from those collected from root-knot nematodes. Importantly, these differences affected the structure of *P. chlamydosporia* populations during the parasitic phase of the fungus. An isolate collected from the root-knot nematode (RKN), *Meloidogyne incognita*, was much more abundant in the rhizosphere of healthy potato and tomato plants as well as RKN infested plants than one collected from potato cyst nematodes (PCN), *Globodera* spp. However, the latter isolate was most abundant in eggs of PCN, which suggested that differential growth of mixed isolates in the rhizosphere might occur during both the parasitic and saprophytic phases of the fungus. The implications of these interactions in the exploitation of *P. chlamydosporia* as a biological control agent are discussed.

Key words: cyst nematodes, root-knot nematodes, *Pochonia chlamydosporia*, intraspecific variation, biological control

Introduction

Selected isolates of the nematophagous fungus, *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*) applied to soil as aqueous suspensions of chlamydospores have significantly reduced populations of root-knot nematodes (*Meloidogyne spp.*) on some crops (Atkins *et al.*, 2003). Although levels of control have been significant (up to 70%), they have not matched those measured in suppressive soils that contain *P. chlamydosporia* (Kerry and Crump, 1998). In these soils, there is much intraspecific variation amongst isolates of the fungus collected from different nematode eggs in the same site. The role of this variation in *P. chlamydosporia* populations in relation to the regulation of host nematode populations is not clear and is an important research topic. As applications based on a cocktail of isolates are likely to be too expensive to produce and register, the commercial development of the fungus is likely to depend on the selection of a single isolate. Biological control agents such as *P. chlamydosporia* are unlikely, therefore, to provide practical rates of control of root-knot nematode populations unless they are used in integrated strategies with other control measures.

Molecular diagnostic methods that distinguish individual isolates of *P. chlamydosporia* have been developed (see Atkins *et al.*, this volume) and will underpin studies of the interactions between isolates in the rhizosphere. The fungus is a generalist, facultative parasite that is able to grow in the rhizosphere of a range of plants and parasitise the eggs of several nematode genera and molluscs and fungal spores (Kerry and Jaffee, 1997). Simple bioassays on water agar in which the eggs of different nematode genera are exposed to different isolates of the fungus have not indicated that individual isolates have host preferences. However,

preliminary results using molecular diagnostic techniques have suggested that individual isolates have more specific interactions with their hosts, which may be obscured on agar when the abundance of the fungus is much greater than that which develops in the rhizosphere. This paper reports on some recent data that has used molecular techniques to study host preference and interactions between selected isolates in the rhizosphere.

Materials and methods

PCR diagnostics of P. chlamydosporia *isolates*

Isolates of *P. chlamydosporia* were freeze-dried and maintained in a collection at Rothamsted Research as a research resource. When required, isolates were revived on agar media for 14 days at 25° C. For DNA extraction, mycelium was collected from 7 -day old Czapek Dox broth cultures that had been inoculated with fungal plugs from the margins of cultures on agar. Genomic DNA was extracted from the mycelium of each isolate as described by Morton *et al.* (2003a) and primers derived from the β-tubulin gene were used in PCR assays to confirm their identity as *P. chlamydosporia* var. *chlamydosporia* (Hirsch *et al.*, 2000). ERIC-PCR was used to distinguish isolates of *P. chlamydosporia* (Arora *et al.*, 1996) and the banding patterns were stained, photographed and analysed as described by Morton *et al.* (2003a). Only bands that were consistent in three independent reactions and between 100bp and 1Kb in size were used in the analysis.

Interactions between isolates of P. chlamydosporia *in the rhizosphere of nematode-infected plants*

Chlamydospores of two isolates of *P.chlamydosporia*, one (isolate S) from root-knot nematodes and one (isolate J) from potato cyst nematodes were produced using standard procedures (de Leij and Kerry, 1991) and applied to a compost (three parts peat compost: one part coarse sand, v/v) at a rate of 5,000 spores g^{-1} soil. Isolates were applied alone or together (a total of 10,000 spores g^{-1} soil) before the compost in each pot was planted with a pre-germinated tomato seedling or a potato chit. Each plant was allowed to establish for 7 days before 2,000 second-stage juveniles of either *M. incognita* or *G. pallida* were applied around the roots of each plant. Plants without nematodes were used as controls and each treatment was performed in triplicate and pots arranged in randomised blocks on the glasshouse bench. The incubation period was completed after 11 weeks, which allowed time for both species of nematode to complete their life cycles, and the nematode population, proportion of infected eggs and the abundance of each isolate in eggs and the rhizosphere were then assessed using standard techniques (Hooper, 1986). The diagnosis and quantification of individual isolates of the fungus was done in a two stage process in which standard diluting procedures were used on a selective medium (Kerry *et al.*, 1993). For diagnosis, the medium was overlain with a cellophane disc and 30, individual colonies were picked from each plate with sterile toothpicks and genomic DNA extracted for PCR analysis as described by Klimyuk *et al.*, 1993. All colonies were removed from plates with < 30 colonies. The cellophane discs prevent contamination of the colonies with agar, which interferes with the DNA polymerase used in PCR.

Results and discussion

Phylogenetic analysis of the data generated by ERIC-PCR indicated some grouping based on geographic origin but the most significant grouping was based on the original host from which the fungus was isolated (Figure 1). Isolates from *Meloidogyne* spp. were distinct from

those collected from cyst nematodes (*Heterodera* spp. and *Globodera* spp.) and those from *Nacobbus aberrans* collected in Mexico. Although *P. chlamydosporia* is a generalist, facultative parasite in soil, it appears that individual isolates of the fungus may exhibit host preferences. In general, the length of branches in the phylogram were longer for fungal isolates from cyst nematodes than from root-knot nematodes. This is a measure of variation between isolates and is probably related to variation in the host: cyst nematodes being mostly amphimictic whilst root-knot nematodes are mostly parthenogenetic. The ERIC patterns for some isolates from widely separated geographic origins could not be distinguished, which may suggest a long association between the host and the pathogen.

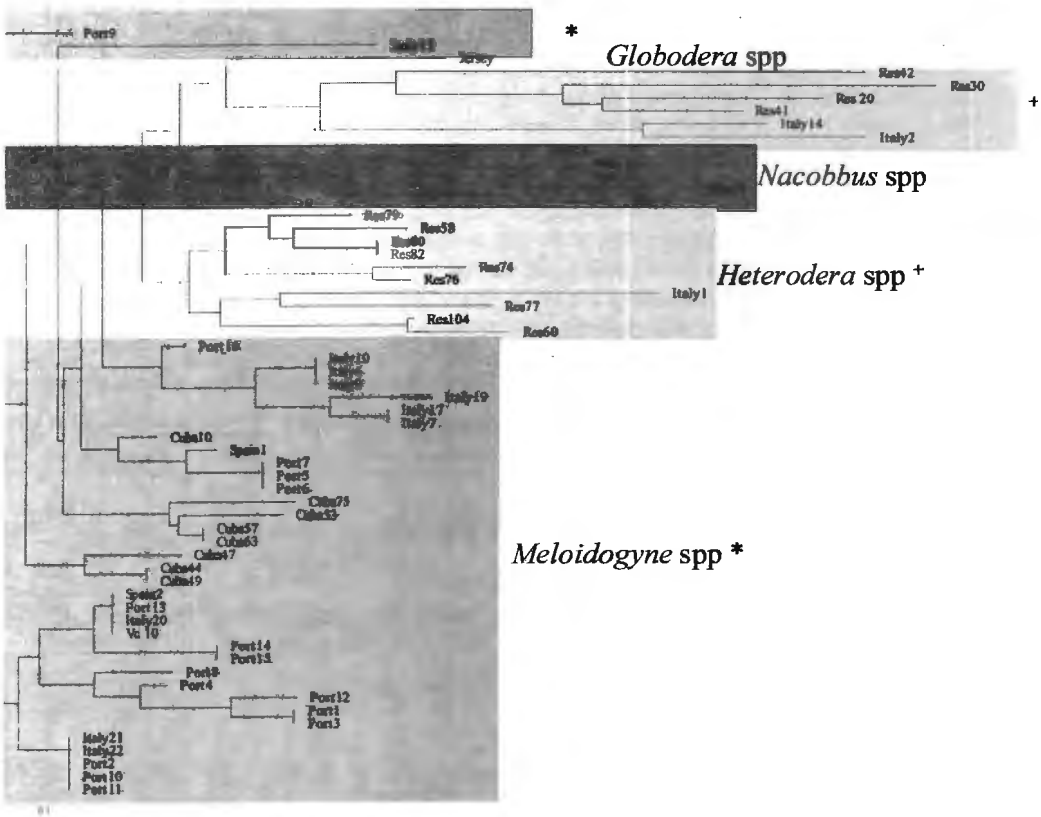

Figure 1. Phylogram showing the genetic variation in isolates of *Pochonia chlamydosporia* from different geographical regions and different nematode hosts as determined by ERIC-PCR. The phylogram is a neighbour-joining tree generated using FreeTree.

An alkaline serine protease has been identified in secretions of *P. chlamydosporia* and was considered to be a virulence and host range determinant (Segers *et al.*, 1994). The enzyme, designated VCP1, from root-knot nematodes removes the outer vitelline membrane of the eggshell of root-knot nematodes and exposes the chitin layer but has little effect on the eggs of the cyst nematode, *G. pallida*. Amino acid sequences of the enzyme from three isolates of

the fungus from cyst nematodes were compared with those from three isolates from root-knot nematodes and consistent polymorphisms between the two types were observed (Morton *et al.*, 2003b). Although these polymorphisms appeared on the rim of the binding region of the enzyme, much work remains before the role of this enzyme in host specificity is fully elucidated. As this enzyme is produced early in the infection process before the eggshell is penetrated, it may prove to be a useful indicator of the change from the saprophytic to the parasitic phase of the development of the fungus.

Only a subset of the data from the mixed fungal isolate treatments in the pot experiment is presented here and a fuller account of the experiment is given by Mauchline *et al.* (2004). The isolate S originally from root-knot nematodes dominated in the rhizosphere of both potato and tomato plants and represented >80% of the fungal population (Figure 2). This isolate was also more abundant ($P< 0.001$) on roots infected with *M. incognita* on both plant hosts but infection with *G. pallida* did not have a significant effect. However, when the infected eggs of *G. pallida* from potato roots were analysed approximately 60% contained isolate J, originally from potato cyst nematodes, even though this isolate represented <20% of the fungal population in the rhizosphere. Too few *G. pallida* eggs were produced on tomato roots for a meaningful analysis on this host. Isolate S that appeared to be more rhizosphere competent than isolate J was found in >80% of the *M. incognita* eggs on potato roots and in all those eggs on tomato roots (Figure 3). It would appear that the differences between isolates observed at the molecular level have implications for the behaviour of their populations and that selection of the fungus occurs during both the saprophytic and parasitic phases of their development. The use of the selective medium and PCR provided a powerful tool for understanding how different isolates interact in the rhizosphere.

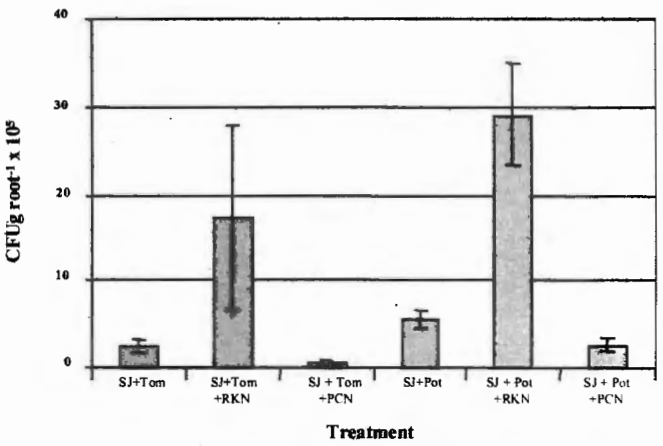

Figure 2. Growth of two isolates of *P. chlamydosporia* in the rhizosphere of plants infected with root-knot and potato cyst nematodes

The differences observed between isolates at the molecular level appear to have implications for the activity of isolates at the population level and the original host from which the isolate was collected clearly affected its performance as a biological control agent. The isolate from cyst nematodes had a significant effect on the numbers of *G. pallida* infected but not on the numbers of *M. incognita* and, similarly, the isolate originally from root-knot

nematodes only significantly infected *M. incognita* eggs. This is the first demonstration of differences in host preference between isolates of *P. chlamydosporia* at the sub-specific level. However, the selection of isolates for the control of particular nematode pest problems will be required and there is a need to develop more sensitive *in vitro* assays, which will enable host preferences to be detected. Assays based on the induction of appressoria on artificial surfaces (Lopez-Llorca *et al.*, 2002) and LD_{50} analyses to quantify virulence are currently being investigated.

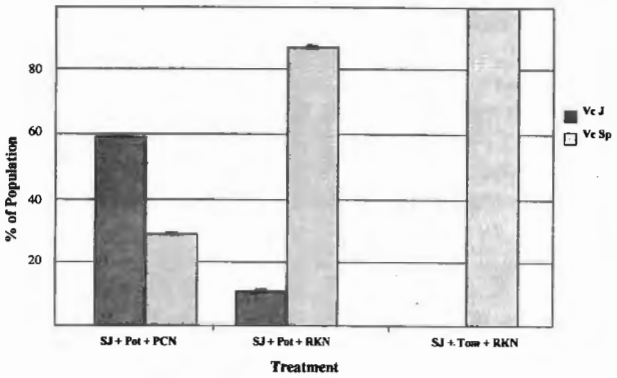

Figure 3. Presence of two isolates of *P. chlamydosporia* in eggs of root-knot and potato cyst nematodes after ten weeks incubation

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Biological control of plant parasitic nematodes with *Paecilomyces lilacinus*, strain 251

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Abstract: Biological control agents are potential alternatives for the chemicals presently used to fight plant pests. To successfully commercialize biological control agents, an advanced technology to produce sufficient numbers of propagules in combination with stable formulation is essential. Due to the phase out of methyl bromide for control of a wide range of soil-borne pests, a potential market for a biological nematicide based on *Paecilomyces lilacinus*, strain 251, has opened up. This fungus has already demonstrated efficacy against root knot, cyst nematodes and other important groups of plant parasitic nematodes. Recent developments in solid state fermentation and formulation technology provide the opportunity to successfully commercialize this biocontrol agent and introduce it into a world wide market.

Key words: biological control, nematodes, solid state fermentation, *Paecilomyces lilacinus* strain 251

Introduction

Environmental and health concerns over the use of some nematicides and the phase out of methyl bromide (Mbr) for soil fumigation has led to an increased interest in the development of alternative strategies for control of plant parasitic nematodes (Kerry, 2001; Maragos, 1999). Farmers heavily depend on Mbr for control of soil borne pests, including plant parasitic nematodes. Due to the phase out of Mbr in developed countries until 2005 and in developing countries until 2015, respectively, alternative control measures are needed in the future (Sikora, 2002). However, despite all research efforts, the commercialization of biocontrol agents has proved difficult. Although some products have been developed, so far none is in widespread use.

Paecilomyces lilacinus (Thom) Samson is a facultative egg pathogen of nematode females and eggs and the most extensively field tested biological control agent for nematodes (Kerry, 1996). Strain 251, deposited at the Australian Government Analytical Laboratory (AGAL; # 89/030550), is currently registered as BIOACT WG and used commercially for control of nematodes on Banana. This strain, isolated from a *Meloidogyne* egg mass, originated from the Philippines and was selected due to his efficacy for control of *Meloidogyne* spp. on tomato and *Globodera rostochiensis* on potato (Davide and Zorilla; 1983, Davide, 1985). Besides the efficacy of *P. lilacinus* against the eggs of cyst and root-knot nematodes, this particular strain has shown also in-vitro activity against juveniles of free-living plant parasitic nematodes such as *Radopholus similis*, *Pratylenchus* spp., *Rotylenchus reniformis* and others (Davide and Williams, 1999). However, although patented and registered as a biological nematicide in the Philippines, production and use of *P. lilacinus* strain 251 has never reached a larger scale.

For some time, *P. lilacinus* strain 251 was licensed to an Australian based company and sold as PAECIL™. The product consisted of the dried bulk fermenter substrate. Besides the problem of the applicator exposure to high numbers of conidia, handling the product was time

problem of the applicator exposure to high numbers of conidia, handling the product was time consuming since the formulation needed to be soaked in water for several hours followed by filtering the suspension through cheese cloth before application. However, due to the lack of production capacity and delays in registration, this product failed although efficacy had been demonstrated in many trials. Table 1 shows one example for a successful field trial for control of root-knot nematodes with *P. lilacinus* strain 251, in comparison to the nematicides Nemacur and Rugby. It could be demonstrated that the application of 2 x 10^9 conidia per plant was sufficient to achieve a reduction of the gall index at 8 weeks after planting, comparable to Rugby. In addition, fruit yield was increased by 92% which was threefold higher compared to Nemacur, but not different from treatment with Rugby.

Table 1. Results of a field trial for control of root-knot nematodes on tomato in Queensland, Australia. (Modified after Holland and Williams, 1998)

Treatment	Rate	Gall index[4]	% fruit yield increase
Control	-	5,1	-
Nemacur[1]	11 kg/ha	5,0	27
Rugby[2]	10 kg/ha	2,9	92
P.lilacinus 251[3]	1.0 g/plant	2,8	88

[1] 100 g Fenamiphos/kg; [2] 100 g Cadusaphos/kg; [3] 1 g contained at least 2 x 10^9 conidia/g; [4] Gall index rating from 0 = nil damage to 10 = all roots severely damaged, evaluated 8 weeks after planting

Besides other factors, commercial success of biocontrol products depends strongly on cost-effective mass production systems, a formulation with sufficient shelf life and high quality (Kiewnick, 2001). Thus, formulation technology is one of the key issues in successful commercialization of biocontrol agents. It has to be considered at all stages from production to its action on the target and can even restore activity or increase efficacy against pathogens (Jones and Burges, 1999).

Currently, *P. lilacinus* strain 251 is licensed by two companies, PROPHYTA Biologischer Pflanzenschutz GmbH (PBP) in Germany and Biological Control Products SA (Pty) Ltd (BCP) in South Africa. Both companies use solid state fermentation for the mass production of aerial conidia, since *Paecilomyces* produces vast amounts of conidia on solid substrates in a short period of time. However, PBP uses a proprietary microscreen technology to separate the conidia after fermentation, followed by fluidized bed drying (Kiewnick, 2001). The final product is a water dispersible granule with a concentration of at least 2 x 10^9 conidia/g product. In contrast, BCP formulates the dry conidia into a wettable powder formulation, containing 1 x 10^9 conidia/g product. Their product *PL*plus needs to be applied in combination with a growth stimulant and can be delivered to the crop as a drench, spray or via drip irrigation. Both products, BIOACT® WG and *Pl*plus, show a sufficient shelf live of at least six months when stored at temperatures below 6 °C (Anonymous, 2002 and 2003). However, freezing of *Pl*plus causes complete loss of viability (Anonymous, 2003) whereas *P. lilacinus* formulated as WG can be stored at –18°C for 12 months without loss of viability (Anonymous, 2002).

Discussion

Paecilomyces lilacinus strain 251 seems to be a good candidate for successful commercialization. In the past, one of the major restrictions for the use of *P. lilacinus* as a biological nematicide was that relatively large dose rates (1 x 10^6 conidia/g soil) are needed for nematode control (Kerry, 1996). However, a cost efficient production technology can reduce the price for a biological control product on a per ha basis significantly (Kiewnick, 2001). Furthermore, most of the hurdles encountered during the development of a biological control product (Lueth, 2000) seemed to be overcome. This indicates that there is a good chance to include this egg pathogenic fungus into an integrated pest management system for the control of plant parasitic nematodes. Field trials in South Africa have already given promising results for the inclusion of *P. lilacinus* strain 251 into an IPM program for control of root-knot nematodes on tobacco (Van Biljon and Botha, 1998). Future research needs to focus on the effects of new developments in fermentation and formulation technologies on the efficacy of this fungal biocontrol agent.

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Risk assessment of fungal biocontrol agents

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Abstract: The development of biological control products faces several obstacles before a successful commercialization is achieved. Despite the high public interest in alternatives to chemical pesticides, the number of biocontrol products, successfully introduced into the market, is still very low. Although biological control products demonstrated their ability to efficiently control pests and diseases without causing any adverse effects to the environment, there are still concerns about the ecological risks. Evaluating the environmental effects (toxicity and pathogenicity) and the fate (dispersal and persistence) of the microbial agents is difficult. In addition, models to appropriately assess exposure are still missing and need to be developed or modified for microbial pesticides. The nematophagous fungus *Paecilomyces lilacinus* strain 251, was chosen as a model organism to identify the parameters needed to predict the fate of fungal biocontrol agents in the environment. Population dynamic of *P.lilacinus* in the soil and rhizosphere was monitored. The effect of application rate, formulation, temperature, method of application and the presence or absence of the target pest as well as the host plant on the persistence of *P.lilacinus* was evaluated. Preliminary results indicate that *P.lilacinus* is not able to multiply in soil or the rhizosphere of tomato plants and is not establishing itself in the environment. When the currently available data are evaluated with the methodology proposed in the ERBIC approach, a very low risk index indicates that no negative effects on the environment are expected by using *P.lilacinus* strain 251 as a biological nematicide.

Key words: *Paecilomyces lilacinus* strain 251, biological nematicide, monitoring, persistence, risk assessment

Introduction

Microorganisms provide a large but untapped source for biological control of pests and diseases. However, the number of successfully commercialized biocontrol products (excluding *Bt* based preparations) is still low due to high costs for product development and the regulatory approvals required for each strain, formulation and intended use (Cook et al., 1996). Although advances in the production and formulation of fungal biocontrol agents have been made (Kiewnick, 2001), regulatory issues are still the main hurdle in product development (Lueth, 2000). In Europe, guidelines on how to appropriately address the risks involved by using a biocontrol agent (BCA) are still incomplete or missing. More research is needed focusing on the development of test systems and guidelines to adequately measure the potential risk a microorganism poses to the environment. In general this risk is defined as the combination of hazard and exposure (Cook et al., 1996). Consequently, when using a BCA with a known hazard, the risk can be reduced by limiting the exposure, and a low risk can be expected by using a BCA with now hazard and high exposure (Cook et al., 1996). Currently, risk assessment is essential for the registration process of biological control products based on microorganisms. One of the most important issues is that BCAs are safe for humans and non-target organisms (De Jong et al., 1999). In addition, the fate and behavior of the organism in the environment (Vänninen et al., 2000), the exposure of the worker or the applicator, the effect of the BCA on beneficial non-target organisms like mycorrhiza (Godeas et al., 1999)

and other microorganisms (Moenne-Loccoz et al., 1998) needs to be addressed in order to register a biocontrol agent. One of the main problems is the lack of acceptable methods that would adequately address and evaluate the above mentioned risks.

Paecilomyces lilacinus (Thom) Samson is a facultative egg pathogen which attacks mainly sedentary stages of plant parasitic nematodes and is by far the most extensively field tested nematode antagonist (BCA; Kerry, 1996). In addition, *P. lilacinus* is considered to be one of the most promising and practicable BCA for the management of plant parasitic nematodes (Siddiqui, 2000). *P. lilacinus* strain 251 is registered as BIOACT® WG and sold in the Philippines for control of nematodes on Banana. It is also currently undergoing registration procedures as BIOACT® WG in Europe and as MeloCon™ WG in the United States. This strain, originally isolated from a *Meloidogyne* egg mass in the Philippines, was selected due to its efficacy against *Meloidogyne* spp. on tomato and *Globodera rostochiensis* on potato (Davide and Zorilla, 1983; Davide, 1985).

The biological nematicide *P. lilacinus* was selected as a model organism for fungal BCA's to assess the possibilities of any adverse effects to the environment by using this antagonist. Environmental fate testing is intended to demonstrate if the BCA is capable of surviving and establishing in the environment. These tests further provide an indication of the extent of the exposure of non-target organisms to the BCA. In addition, information on the human health risk for fungal BCA's is essential for an adequate risk assessment of fungal biocontrol agents.

Material and methods

Persistence

To evaluate the risk involved with the application of a fungal biocontrol agent, the fate and behavior of the introduced organism needs to be monitored. The common method for monitoring a fungal biocontrol agent is soil dilution plating onto semi-selective media. Although several media have been described for the enumeration of colony forming units (cfu) of *P. lilacinus*, those media were not suitable for isolation of this particular strain 251 from soil samples. For isolations from greenhouse experiments, OHIO agar, a common medium for the isolation and enumeration of soil-borne fungi (Johnson and Curl, 1972), was chosen. For isolations from field plots, a modified OHIO agar was utilized (Kiewnick et al., 2003). With this modified medium, population densities as low as 1×10^3 cfu of *P. lilacinus* per gram of soil can be quantified (data not presented). For pot experiments under greenhouse conditions, the formulated product containing 1×10^{10} viable conidia/g was suspended in tap water and incorporated into field soil/sand mix (1:1, v:v). The rate of 0,2 g/pot resulted in initial concentrations between LOG 6,16 and LOG 6,23/g soil. In addition, for the treatment with the combination of tomato (cv. Hellfrucht) and nematodes, 5000 eggs of *Meloidogyne hapla* were incorporated into the substrate. Untreated substrate served as control for evaluation of the background level of other filamentous fungi. Pots were left in the greenhouse for 6 days and watered if necessary. At day 7, tomato plants (6 weeks old) were transplanted into the pots. Soil samples were taken for up to 98 days past application from either the bulk soil or the rhizosphere from tomato plants followed by dilution plating on OHIO agar.

To evaluate the persistence of *P.lilacinus* formulated as WG under field conditions, BIOACT® WG was applied with regular spraying equipment at a rate of 4 kg/ha and immediately incorporated into the soil (7-9cm deep). Samples from 20 subplots were taken one hour after application and dilution plated onto modified OHIO agar.

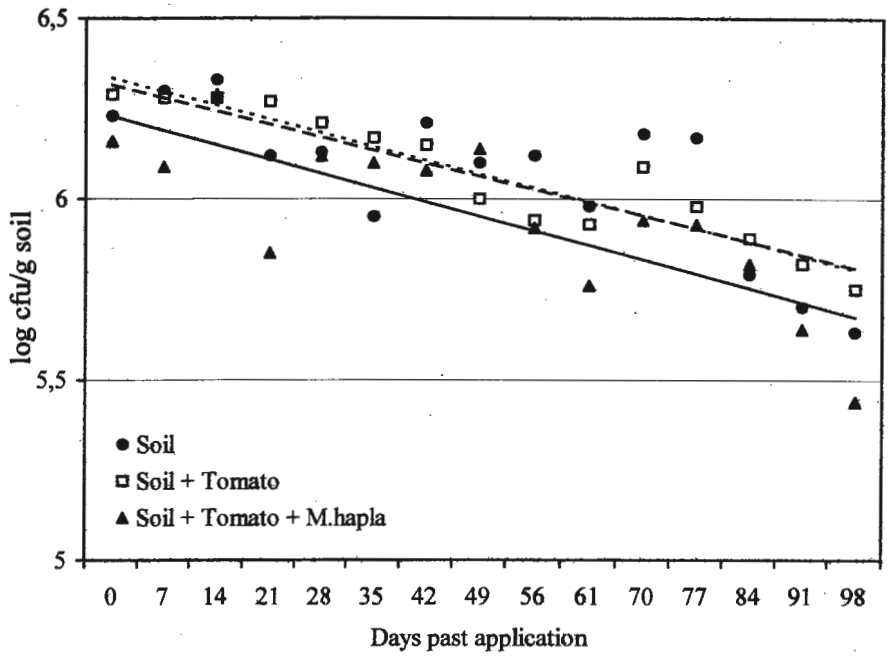

Figure 1. Effect of soil (r^2 = 0,801***), soil plus tomato (r^2 = 0,812***) and soil plus tomato and *Meloidogyne hapla* (r^2 = 0,536***) on the persistence of *P. lilacinus* formulated as WG under greenhouse conditions (n=3).

Effect of temperature

The microbial human health risk assessment of fungal biocontrol agents is one of the major concerns during the registration of biocontrol products. The ability of a biocontrol agent to grow at temperatures of 36 °C or higher is, besides other tests, one indicator for a possible pathogenicity towards mammals. Determination of the maximum growth temperature for radial growth is currently used to evaluate the risk for fungal biocontrol agents. However, since most products contain formulated conidia or blastospores as active ingredient, these tests should be performed with the formulated products.

Conidia of *P. lilacinus* from the formulated product were plated on Sabouraud dextrose agar (SDA) and exposed to 36°C degrees followed by determination of i) the germination rate after 24 and 48 hours ii) germ tube development iii) the survival rate depending on the exposure time.

Results and discussion

Persistence

The data presented in figure 1 demonstrate that the presence of the host plant and the target nematode, *M. hapla*, did not significantly affect the decline of the *P.lilacinus* concentration in the soil. Linear regression analysis resulted in highly significant correlation coefficients for the soil (r^2 = 0,801***), soil plus tomato (r^2 = 0,812***) and soil plus tomato plus *M.hapla* (r^2 = 0,536***). Additionally, statistical comparison of the slopes revealed that there was no significant difference (p=0,05) between the treatments. This leads to the conclusion that

neither the decline of the *P.lilacinus* population in the soil is affected by the presence of the host plant and the target nematode nor does *P.lilacinus* multiply in the rhizosphere of tomato plants.

Results from growth chamber and greenhouse studies indicate that the persistence of *P. lilacinus* strain 251 seems to be predictable. In none of the conducted experiments an increase in the population density was observed. This effect was independent from the presence of the target, root-knot nematodes, or the host plant tomato.

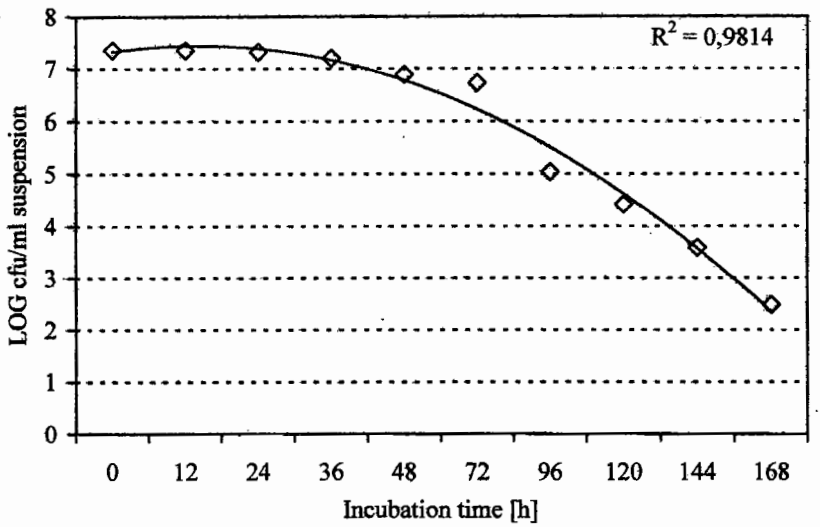

Figure 2. Effect of exposure to 36°C for up to 168 hours on the number of surviving colony forming units (cfu) of *Paecilomyces lilacinus* strain 251 formulated as WG

Under field conditions, the spatial distribution of *P.lilacinus* determined immediately after application and incorporation varied greatly (Kiewnick et al., 2003). The concentration of cfu/g soil ranged between 2,5 x 10^4 and 1,4 x 10^5, which was 12 to 2-fold lower than the expected concentration of 3 x 10^5 /g soil. However, these initial data indicate that the persistence of *P.lilacinus* in the environment is expected to be rather low. Repeated sampling was done every 30 days for the whole cropping season (data not presented) and confirmed a decline of the *P.lilacinus* population of more than 95% (Kiewnick & Roumbos, 2003; unpublished data).

Effect of temperature

As most biocontrol fungi *P.lilacinus* (strain 251) showed no radial growth at 36°C (data not presented). In contrast, the germination of the conidia from the formulated product was only slightly delayed at 36°C compared to the control at 24°C (Kiewnick et al., 2002). However, when the germ tube development was measured over a period of 168 hours, it could be demonstrated that after 72 hours of exposure or more, the germ tubes showed deformations and stopped growing (Kiewnick et al., 2002). Additional staining with fluorescein diacetate

and observation under UV-light revealed that those fungal structures showing deformations and lysis were no longer viable (data not presented). To verify that conidia, when exposed to 36 °C, were not able to survive, the number of cfu per plate after incubation for up to 168 hours was determined. For this test, up to 2 x 10^6 conidia were spread on SDA plates using a spiral plater. After exposure between 96 and 168 hours a rapid decline in the cfu number was found indicating that although germination was still possible, *P.lilacinus* could not survive at 36°C (figure 2).

ERBIC approach

Van Lenteren and co-authors (2003) developed a methodology for the risk assessment of import and release of exotic natural enemies used in inundative forms of biological control. ERBIC stands for "Evaluating environmental risks of biological control introductions into Europe". This approach integrates information on the potential of a BCA to establish, disperse, the host range and possible direct and indirect effects on non-target organisms (Van Lenteren et al., 2003).

Table 1. Risk assessment based on the *ERBIC* approach for *Paecilomyces lilacinus* stain 251 and commercially available inundative BCA's (modified after Van Lenteren et al., 2003)

BCA		Establishment[3]	Dispersal[4]	Host range[5]	Direct effects[6]	Indirect effects[6]	Risk index SUM[7]
Beauveria bassiana	L[1]	2	2	5	5	3	
	M[2]	1	1	5	3	2	
	LxM	**2**	**2**	**25**	**15**	**6**	**50**
Metarhizium anisopliae	L	3	1	5	1	1	
	M	5	1	5	1	1	
	LxM	**15**	**1**	**25**	**1**	**1**	**43**
M.anisopliae var. acridum	L	4	4	3	4	2	
	M	2	2	5	3	1	
	LxM	**8**	**8**	**15**	**12**	**2**	**45**
Steinernema feltiae	L	3	1	5	4	4	
	M	5	1	5	2	1	
	LxM	**15**	**1**	**25**	**8**	**4**	**53**
Paecilomyces lilacinus **251**	L	2	1	3	1	1	
	M	1	1	4	1	1	
	LxM	**2**	**1**	**12**	**1**	**1**	**17**

[1] likelihood; [2] magnitude; [3] establishment in non-target habitat; [4] distance moved and % BCA dispersing from release area; [5] ecological host range and taxon range, attacked by the BCA; [6] mortality, population suppression or local extinction of non-target organism; [7] Risk index total, ranging from 5 (no risk) to 125 (maximum risk).

In the ERBIC approach, risk is defined as the result of the likelihood times the magnitude of the possible effects on non-target organisms. The assessment is divided into three steps: 1) Risk identification and evaluation procedure; 2) Risk management plan dealing with the reduction of possible risks and 3) Risk/benefit analysis of current and new alternative pest management methods. For the calculation of the overall risk value for a BCA, individual risk

values of the likelihood as well as the magnitude for establishment, dispersal, host range and direct and indirect effects on non-targets are calculated (Van Lenteren et al., 2003). Individual risk values range between 1 (very unlikely) and 5 (very likely) for likelihood and between 1 (minimal) and 5 (massive) for the magnitude of the above mentioned parameters. Table 1 shows the risk indices based on the ERBIC system for commercial available, inundative BCA's (Eilenberg et al., 2001) in comparison to *P.lilacinus* strain 251.

Based on the currently available data, the risk assessment methodology proposed by Van Lenteren et al. (2003) was applied to *P.lilacinus* 251 (table 1). Concerning the establishment of *P.lilacinus* in non-target habitats, the values for likelihood and magnitude are 2 and 1, respectively. This is based on a low to moderate persistence and a low competitiveness of *P.lilacinus* towards other soil microorganisms (Kiewnick et. al., 2003; Khan et al., 2003). The potential for dispersal from a treated area is negligible, since *P.lilacinus* is applied to and incorporated into the soil. This parameter refers more to classical biological control using predatory insects or mites. Biological insecticides such as *B. bassiana* and *M. anisopliae var. acridum* receive higher values for potential dispersal when applied as spray in open fields. Infected insects could carry the organism away from the treated area, causing dispersal of the BCA (table1). Concerning the ecological and the taxon host range attacked by *P.lilacinus*, the methodology is clearly overestimating the potential risks. Once *P.lilacinus* is applied to the soil, the propensity to realize its ecological host range is a maximum of 4 to10 species of plant parasitic nematodes. In addition, considering that the range of plant parasitic nematodes *P.lilacinus* is able to attack are within the order Tylenchida, the magnitude of this parameter (value 4) is not applicable to biological nematicides and needs to be replaced. Based on the currently available data, no direct or indirect effects on non-target organisms are expected once *P.lilacinus* is applied. Due to the envisaged use, toxicological studies (EPA, 2003) and the lacking ability for production of toxins or antibiotics (Khan et al., 2003), no direct or indirect side effects on non-target organisms are expected. In total, the calculated risk index for *P.lilacinus* is 17. In comparison to biological insecticides and the entomopathogenic nematode *Steinernema feltiae*, the ERBIC model estimates the potential risks as very low. Van Lenteren et al. (2003) considered only 7 out of 29 biocontrol organisms (6 parasitoids and 1 predatory mite) to be safer than *P.lilacinus*. However, the basis for this classification is not applicable for fungal biocontrol agents in general and needs to be modified for each individual BCA.

In conclusion, the presented data on the possibility of *P. lilacinus* strain 251 to establish and persist in the environment and the evaluation of the microbial human health risk by determination of growth and survival at 36°C, indicate that this fungal biocontrol agent is posing no or only a low risk to the environment once it is applied. However, more information for the appropriate risk assessment of BCA's is needed and new, specific guidelines for testing microbial pesticides have to be developed in the future. Experiments are underway to gain more information on the long term survival under greenhouse and field conditions. Based on these data, appropriate models to predict the fate of *P.lilacinus* in the environment can be developed. In addition, the exposure of workers and bystanders, when handling and applying fungal biocontrol products has to be addressed.

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Biocontrol activity of phenazine-producing rhizobacterium *Pseudomonas chlororaphis* 44

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Abstract: Plant associated fluorescent pseudomonas strains, which can colonize roots of crop plants and produce a wide range of antifungal metabolites represent a real alternative to the application of chemical fungicides. *Pseudomonas chlororaphis* (= *aureofaciens* 449) isolated from rhizosphere of maize suppresses numerous plant pathogens *in vitro*. The strain was found to produce three phenazine antibiotics (PCA, 2-OH-PCA, 2-OH-PHZ), hydrogen cyanide, siderophore(s) and exoprotease(s). It also produces at least three types of regulatory quorum sensing N-acyl-homoserine lactones (acyl-HSLs) signal molecules: N-butanoyl-HSL, N-hexanoyl-HSL and N-(3-oxo-hexanoyl)-HSL. Mini-Tn5-mutants of strain 449 deficient in PCA production were isolated. Two of them were compared with the parental strain for ability to protect beans and cucumbers against *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, respectively, under greenhouse conditions. Treatment with the parental strain decreased the incidence of the diseases caused by these fungi by 70% and 50%, respectively. Contrary to that, both mutants were unable to control the pathogens. Thus, indicating the critical role of phenazines in the inhibition of these fungi.

Keywords: biological control, phenazines, quorum sensing, *N*-acyl homoserine lactone, *Rhizoctonia solani, Sclerotinia sclerotiorum*

Introduction

Biological control of plant pathogens based on natural interactions between organisms offers a potential means of overcoming the ecological problems induced by chemical pesticides and a model for the study of communication among multiple organisms. Certain strains of root colonizing fluorescent *Pseudomonas* spp. produce broad-spectrum antibiotic metabolites that can provide protection against soilborne root diseases. One such class of antibiotics, the phenazines, encompasses a large family of heterocyclic nitrogen-containing pigments. Almost all phenazines exhibit broad-spectrum activity against various species of fungi and bacteria.

The strains producing phenazines can be used as a biocontrol agents; the well-known examples are *P. fluorescens* strain 2-79 and *P. chlororaphis* (=*aureofaciens*) strain 30-84, which are used to protect wheat from take-all disease, caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow & Weller, 1995). The phenazine biosynthetic loci from *P. fluorescens* 2-79 (Mavrodi et al., 1998) and *P. chlororaphis* 30-84 (Delaney et al. 2001) are highly conserved; each contains a seven-gene core operon. Successful biocontrol requires the sending and receiving of signals between the biocontrol agent and the pathogen, plant host, and microbial community surrounding the host (Pierson et al., 1998). The synthesis of phenazine antibiotics in strain 30-84 has been demonstrated to be regulated by a PhzI/R quorum-sensing signaling system that utilizes the acyl-HSL molecule *N*-hexanoyl-L-homoserine lactone (HHL) (Wood et al., 1997).

In this paper we describe strain 449 of *P. chlororaphis* isolated from rhizosphere of maize in Kiev region (Ukraine), able to produce a set of phenazines and provide evidences that these antibiotics are responsible for the bacterium antifungal activity *in vitro* and ability to protect plants against soil-born and foliage diseases caused by *R. solani* and *S. sclerotiorum*, respectively. Strain 449 produces at least three types of acyl-HSLs, however the specific role of these signal molecules in this bacterium biocontrol activity needs to be further elucidated.

Materials and methods

Analysis of phenazine compounds

Phenazine compounds were extracted as described earlier (Delaney et al., 2001) and were analyzed by high pressure liquid chromatography (HPLC) with a reverse- phase C18 column, eluted by a linear gradient to 100% ACN-0.1% TFA at a flow rate of 1.0 ml/min. Antibiotics adsorption was monitored with diode array detector (UV6000) at wavelength in range of 250-450 nm. Phenazine compounds were identified by retention time and UV spectrum.

Transposon mini-Tn5 mutagenesis and mutants characterization.

A spontaneous mutant of strain 449, resistant to rifampicin (Rif^r) was used as a recipient in mating on membrane filters with *E. coli* S17-1 carrying the pUT-miniTn5-Km2 plasmid performed generally by a procedure described (de Lorenco and Timmis, 1994). Phz$^-$ mutants were identified initially by alteration or loss of colony pigmentation on LB-agar. Changes in phenazine production were confirmed by HPLC analyses. In order to determine the direction and localization of the miniTn5 transposon insertion by PCR analysis we used *phzF* forward primer 5-AAGCGTTCCAGCAGCCTCAATG-3', and miniTn5Km2 reverse primer 5'-CCATTCTCACCGGATTCAGTCG-3' designed to published sequence of genes *phzF* (GeneBank database AF195615) and kan gene (GeneBank database U32991), respectively.

Acyl-HSL on plate assay and identification of the acyl-HSLs by TLC

Acyl-HSLs bioassays were performed on plates and by TLC analysis using pigment violacein-reporter strain *Chromobacterium violaceum* CV026, ∃-galactosidase-producing reporter *Agrobacterium tumefaciens* NTL4/pZLR4, and three *E. coli* strains carrying recombinant plasmids pSB401, pSB536 or pSB1075 designed for *lux*-based reporter assays (McClean et al., 1996; Shaw et al., 1997; Winson et al., 1998). Ethyl acetate extracts of acyl-HSLs from supernatant of the tested strains were prepared as described (Shaw et al., 1997). Culture supernatant extracts and synthetic acyl-HSL standards were spotted onto glass-backed RP18 reverse-phase TLC plates (Merck, Germany). Samples were separated using 60% (vol/vol) methanol in water as the solvent. Additionally, the presence of gene *phzI* in strain 449 genomic DNA was tested by PCR with primers designed to corresponding gene of strain 30-84 (Genbank no. L33724).

Greenhouse experiments

Protection of beans (cv. "Wax yellow") against *R. solani* root rot disease and of cucumber (cv. "Cfir 413") against *S. sclerotiorum* white mold disease on leaves by strain 449 was performed as described earlier (Chernin et al., 1995; Kamensky et al., 2003).

Results and Discussion

Production of phenazine compounds

Three phenazine antibiotic were detected in extracts from cultures of strain 449 analyzed by HPLC. Each compound was purified by HPLC, and its UV-visible absorption. Spectra for

these compounds closely matched those reported for PCA, 2-OH-PCA and 2-OH-Phz (Fig. 2) described earlier (Delaney et al., 2001).

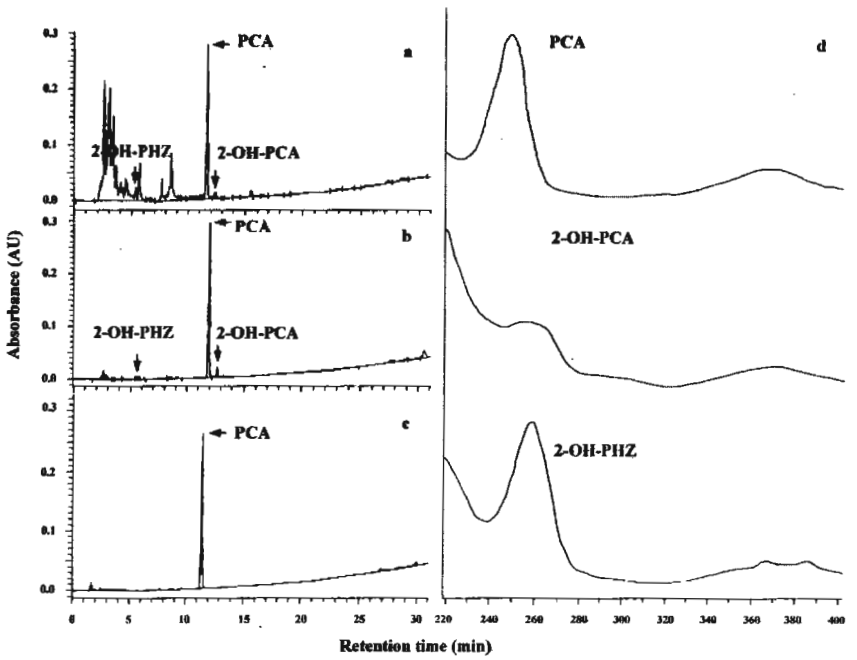

Figure 1. HPLC analysis of phenazine compounds produced by strains *P. a.* 449 (a), *P. c.* 30-84(b), *P. fl.* 2-79(c). *P.c.* strain 449 produces three phenazine antibiotics – phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxyphenazine (2-OH-Phz), The elution profile at the indicated retention time was observed at wavelength 248 nm (specific for PCA absorption) and the peak identity of PCA, 2-OH-PCA and 2-OH-Phz was confirmed by spectral analysis (d) at wavelength (nm) indicated. Retention times for PCA and 2-OH-PHZ are 14.1 and 11.4 min, respectively. Absorption maxima for PCA are 248 and 371 nm. Absorption maxims for 2-OH-PCA are 257 and 369 nm. Absorption maxims for 2-OH-Phz are 257, 368, and 387 nm.

Isolation of Phz⁻ mutants

Km^r exconjugants of strain 449 were recovered at a frequency of ~ 5 x 10^{-6} per initial recipient after mutagenesis with mini-Tn5. Three Phz-deficient non-pigmented mutants were finally selected. Data of HPLC analysis of the two mini-Tn5 mutants, designated as 449::miniTn5#3 and 449::miniTn5#14, showed that these mutants are deficient in production of PCA and consequently two other phenazine antibiotics (2-OH-PCA and 2-OH-PHZ) derived from PCA as a precursor (data not shown). According the size of the PCR products we have calculated the localization of the inserts in positions 1728 and 1882 in gene *phzA* close to its junction to gene *phzB*, both encoding biosynthesis of PCA.

Ability of strain* P. chlororaphis *449 to suppress* R. solani *and* S. sclerotiorum *under greenhouse conditions.

Strain 449 decreased *R. solani* incidence on beans by 70% and *S. sclerotiorum* incidence on cucumbers by 50%. Contrary to that, both mini-Tn5-mutants deficient in phenazine production were unable to control the pathogens (Table 1).

Table 1. Protection of cucumber toward *R. solani* and *S. sclerotiorum*

Strain/Fungus*	*R. solani*	*S. sclerotiorum*
P. c. 449	31.8±8.9a**	52±3.7a
P. c. 449::miniTn5#3	99.3±0.7b	96±4b
P. c. 449::miniTn5#14	85.8±4b	92.3±7.6b

*Disease incidence refers to 100% disease control (the average actual disease control in the experiments was 86.7 ± 12.6 for *R. solani* and 66 ± 10.7 for *S. sclerotiorum*). **Different letters in the same row indicate significant differences between means using the All Pairs Tukey-Kramer test ($\alpha = 0.05$, $p < 0.001$)

Identification of the N-acyl-homoserine lactones produced by* P. chlororaphis *449

Strain 449 strain primary selected as quorum-sensing positive using the plate assay with *C. violaceum* CV026 and *A. tumefaciens* NTL4/pZLR4 bioreporters was subjected for further analysis in aim to identify the individual acyl-HSL signals. TLC analysis of ethyl acetate extracts from supernatants of strain 449 revealed two spots similar to those observed in supernatants of strain 30-84 with Rf value around 0.60 and 0.37 corresponding to N-butanoyl-HSL (BHL) and N-hexanoyl-HSL (HHL) standards. However, in addition to these compounds strain 449 was found produced N-(3-oxo-hexanoyl)-HSL (OHHL) not observed in culture medium of strain 30-84 (fig. 2). By PCR analysis using the pair of primers designed to gene *phzI* encoding acyl-HSL-synthase in strain 30-84 (Genbank no. L33724) we have shown the presence of the similar gene in genomic DNA of strain 449. The PCR products of 734 bp was cloned and sequenced. The sequence revealed 93.6% similarity with gene *phzI* from strain 30-84. So, despite of different origin strains 30-84 and 449 revealed the same pattern of inter-cellular signaling which play a role, as was shown for strain 30-84, in its rhizosphere competence and colonization of host plant roots in natural soil.

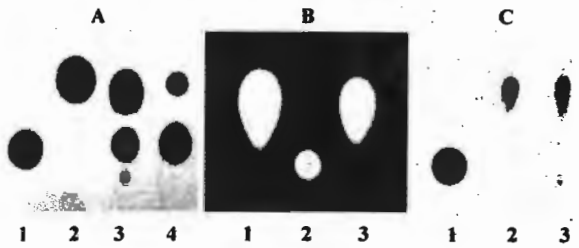

Figure 2. Thin-layer chromatogram of acyl-HSLs extracted from cell-free culture supernatants of strain *P. chlororaphis* 449. TLC plates were overlaid after chromatography with strains CV026 (A), S17-1/pSB401 (B) and NTL4/pZLR4. A: 1- HHL, 2-BHL, 3-strain 449, 4-*P.c.* 30-84 (used as a control); B: 1-OHHL, 2- HHL, 3- *P.c.* 449; C: 1-HHL, 2-OHHL, 3- *P.c.* 449. Acyl-HSLs were located as purple spots against a white background in case of CV026 reporter (A), by bioluminescence detected with bioluminometer in case of *E. coli* strains carrying *lux*-reporter (B) and as blue spots in case of *A. tumefaciens* reporter (C).

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Preventive plant health management: Modern horticulture becomes high tech

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Key words: DNA array, multiplex pathogen detection, disease suppression, biological control, preventive plant health management

Introduction

Scientia Terrae Research Institute (STRI) was founded in 2001 as an independent, not-for-profit research, service, and education center. It actively cooperates with government, academia, industry, growers, and society to seek, develop and disseminate resources and realistic solutions to meet the current challenges to managing plant and environmental health and improving food quality and safety. To accomplish this, STRI is comprised of four separate yet integrated operating units: Plant and Pest Diagnostics, Research and Development, Extension and Education, and Consulting and Professional Services. Together they provide STRI clientele and partners a centralized point for diagnostic assistance, needs assessments, exploratory and developmental research, advice, and other resources. Each unit is briefly described below:

1. Plant and Pest Diagnostics: provides growers and the food sector clinical diagnostic services and advice for preventing and remediating plant health and food safety concerns;
2. Research and Development: works with industry and grower organizations, government agencies, companies, and academic partners on projects with careful regard to promoting economic viability and social responsibility;
3. Extension and Education: serves as a centralized resource for informing and educating university students, growers, food industry personnel, and the public about classical and state-of-the-art integrated crop production practices, and food, plant and environmental microbiology;
4. Consulting and Professional Services: offers regulatory review services to help clients determine feasibility, risks, liabilities, legality, and deliverability of potential offerings to the market.

The remainder of this paper will focus on research and development efforts of STRI and will include key examples of current projects that utilize high technology for horticulture.

Research and Development at STRI

STRI is positioned at the interface between academia and the horticulture industry and food sectors. Its mission is to translate fundamental science into practical, real-world solutions. STRI is guided by the premise that these solutions must be effective, sustainable and mutually beneficial to growers, industry, government, and society. All research and development activities of STRI revolve around the concept of preventive plant health management

(PPHM). In this framework it is important to consider all factors that affect plant health when defining PPHM strategies for crops. Use of certain factors may begin with detection of plant health threats, while other key factors may be optimized to diminish or eliminate plant health risks, thus precluding curative treatments.

The Research and Development Unit consists of four programs that share some overlap in their research areas:

1. Molecular Microbial Diagnostics: pursues and develops new molecular-based approaches for rapid and accurate analysis and assessment of soils, plants, irrigation systems, water supplies, air, and other niches for the presence of specific pathogenic and beneficial microorganisms. These techniques are then further adapted for high-throughput analyses for use in well-equipped clinical as well as less-sophisticated practical laboratory settings.
2. Microbial Ecology and Biological Control: studies the interactions between plants, microorganisms, and the environment. More specifically, it focuses on how microorganisms affect plant health and environmental quality. Its primary function is to transfer this knowledge to the development of reliable, sustainable, and ecologically sound alternatives to conventional crop production practices and environmental protection and remediation strategies.
3. Microbial Food Quality and Safety: identifies critical microbial factors in fresh produce production and processing that affect food quality and safety. It also works with fresh market growers, handlers, and processors to develop effective and advisable quality standards for fruits and vegetable production. Furthermore, it evaluates innovative human pathogen detection techniques and sanitation approaches needed to assure consumers high-quality, safe produce.
4. Product Composition, Assessment and Utilization: examines the control, characterization and potential for modification of the quality, intrinsic characteristics and effects of both primary raw materials and organic by-products that offer potential to be used as secondary raw materials. The ultimate goal of thorough characterization and physico-chemical or microbial modification of these materials is to enhance their functionality and increase their value. More specifically, this research focuses on organic products, in both raw and finished forms, that are being used by or produced in the primary sector (i.e. agriculture and horticulture).

Two research projects are currently underway that span the STRI research and development spectrum. One focuses on rapid detection and quantification of the most economically important soilborne fungal pathogens of vegetable production. The other concentrates on identification of the most important parameters that affect suppressiveness of a disease-suppressive microorganism (DSM) combination in potting media to two different damping-off diseases and on development and verification of a model system for consistent disease suppression. Both of these projects are co-sponsored by the Flemish government and industry partners and are described in the following two sections.

Development of rapid and objective host-specific diagnostic kits for qualitative and quantitative multiplex detection and identification of soilborne plant-pathogenic fungi

A major challenge to integrated disease management is the ease with which plant pathogens are detected and identified. Currently, rapid, simple, accurate, objective and reliable methods do not exist for multiplex pathogen detection and quantification. Conventional procedures are time consuming and laborious, require extensive knowledge of classical taxonomy and culture methods, and typically exclude non-culturable microorganisms. Molecular detection

and identification methods can generate accurate results rapidly enough for use in disease management decisions, but most current techniques allow only one or a few species to be detected per assay. DNA array technology represents a novel solution to all of these concerns.

The purpose of this project is to develop a DNA macroarray to specifically detect and identify economically important plant pathogens in various simple and complex biological matrices. The following results have been realized since the project began in 2001:

1. A general procedure was developed for extracting DNA from several diverse matrices.
2. Highly discriminatory genus- and species-specific oligonucleotide detector probes were designed, selected, and verified for target and related fungi and oomycetes based on aligned internal transcribed spacer (ITS) region sequences of isolates received from several international collections. Currently, 7 genus- and 18 species- specific fungal probes exist on the macroarray, while one oomycete-, 2 genus-, and 15 species-specific oomycete probes are present on the same array.
3. The detection limit of the array was determined to be less than 1 pg DNA for most oligonucleotide detector probes.
4. The detection protocol was optimized to return results within 36 hours of receiving samples.
5. Detection of mixes of fungal and oomycete species in various matrices (i.e. plant tissue, soils, potting media, stonewool, nutrient solutions and water) was achieved repeatedly.
6. Detection of fungal and oomycete species in various matrices was validated with conventional techniques by a clinical diagnostic laboratory on samples originating in the laboratory and from grower environments.

Together, these results demonstrate that the DNA macroarray technology developed herein is: a) specific and highly selective, b) rapid, c) ideal for routine analysis of samples from several different matrices, and d) an approach that facilitates highly sensitive, multiplex detection of important soilborne pathogens.

Presently, experiments are being conducted to compare culture-based enumeration (where possible) and quantitative real-time PCR results to pathogen DNA amplicon hybridization signals on the array for developing the quantitative function of the macroarray. In addition, the current array is being expanded to include economically important bacteria and plant-parasitic nematodes.

Establishment of a model biocontrol-agent based system for reliable and consistent suppression of soilborne diseases of horticultural crops

Other major challenges to integrated disease management include general social position against pesticide use, the loss of effective conventional pesticides, and the lack of efficacy and reliability of biocontrol strategies intended to replace chemical pesticides. Since DSMs are living, their activities are impacted by many factors that pose little or no threat to chemical pesticide efficacy.

It has been demonstrated that substrate conditions such as microbial carrying capacity greatly influence the establishment, survival, and efficacy of specific DSMs as well as natural DSM communities. DSM-mediated disease suppression is generally not widely practiced or accepted by growers worldwide. Although there may be many causes for these circumstances, the most important contributing factors are that DSMs and their suppressive activities are unreliable and are greatly influenced by the conditions under which they are deployed. Therefore, it is necessary to identify the parameters that govern the establishment, longevity, efficacy, sustainability, and limits of DSMs *before* bringing them to market.

It is impractical to study all aspects of each DSM under development or on the market and still provide growers with timely, suitable alternatives to conventional fungicides. However, new high-throughput techniques, instrumentation, empirical and fundamental insights, and international collaborative efforts offer researchers the capacity to expand examination of DSM systems to include more analyses in their work, thus facilitating simultaneous identification of broader sets of potentially relevant factors. Proper analysis and application of these results will lead to more responsible marketing, better quality control, and more effective application of DSM formulations and systems.

The overall goal of this project is to define the parameters that are essential to consistently formulate a DSM-fortified organic substrate that effectively suppresses Pythium and Rhizoctonia damping-off diseases of horticultural crops. The project aims to establish a set of chemical, physical, organic and biological characteristics of organic amendments and substrates that indicate or affect their abilities to support suppression of the two diseases by a DSM combination of *Trichoderma hamatum* 382 and a bacterial DSM strain. Organic amendments to be examined will include several different composts and organic by-products of horticulture and food production.

The project is in its first phase where new methods are being developed for more rapid and robust routine measurements. These new methods include: a) real-time quantitative PCR for specific quantitation of DSMs in various matrices, b) sonic sieving for more rapid separation and less destructive recovery of particulate organic matter fractions than wet sieving, and c) total organic nitrogen analysis of solid and liquid samples to be conducted rapidly and concurrently with combustion-based total carbon, total nitrogen, and total organic carbon analyses.

The project's second phase will focus on how suppressiveness of DSM-fortified substrates is related to or affected by chemical, physical, organic (microbial activity and carrying capacity), and biological (DSM and pathogen colonization and overall microbial community) factors as well as plant responses (plant biomass generation and nutrient uptake). The speed and quality of measurements provided by the latest instruments and analytical methods permit examination of a more complete spectrum of factors in single experiments rather than having to assemble results obtained from different studies to draw conclusions. In addition, a time-series function is included in the experimental design employ the temporal dynamics and biodegradation of organic components to help isolate critical parameters.

In the final phase, a model DSM-fortified organic substrate system will be devised using information gathered and analyzed from the previous phase, and will verify whether this system can be used to select organic materials and consistently formulate effective DSM-fortified organic substrates. Ultimately, this model system could be used to help develop other systems for suppression of soilborne diseases in greenhouse and field settings.

Discussion

Developments from these and other projects at STRI do not directly place high-tech tools into the hands of horticultural producers. Rather, these tools will be used by those who assist growers, whether they provide services, products, or research that directly impact grower decision-making, productivity, profitability, and product quality. STRI is ideally poised at the interface between academia and industry to address the needs of horticulture and the food sector and to continuously move science into solutions.

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Bacterial life inside and outside potato roots and leaves

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Abstract: To study the effect of microenvironments on potato-associated bacteria, the abundance and diversity of bacteria isolated from the rhizosphere, phyllosphere, endorhiza and endosphere of field grown potato was analyzed. A total of 2,648 bacteria isolated during a period of two successesive seasons at three different growth stages of potatoes was screened for antagonism towards the plant-pathogenic fungi *Verticillium dahliae* Kleb. and *Rhizoctonia solani* Kühn. Antagonistic isolates were identified by FAME and by sequencing the bacterial 16S rDNA. Altogether, 41 antagonistic species were identified with 21 of them being typical colonizers of endophytic microenvironments. Richness and diversity indices confirmed the specificity of the microenvironments. All 349 antifungal antagonists were characterized by testing their antagonistic mechanisms and plant growth promoting mechanisms *in vitro*, including glucanolytic, chitinolytic, pectinolytic, cellolytic, and proteolytic activity as well as siderophore and auxin production. Evaluation resulted in the selection of five promising candidates from each microenvironment for biological control.

Key words: antagonistic potential, biological control, endophytes, plant-associated bacteria, soil-borne pathogens, *Verticillium dahliae*, *Rhizoctonia solani*

Introduction

The study of plant-associated bacteria and their antagonistic potential is important not only for understanding their ecological role and the interaction with plants but also for the finding of new biological control agents and the isolation of active compounds (Weller 1988; Emmert & Handelsman 1999; Bloemberg & Lugtenberg 2001).

Verticillium dahliae Kleb. and *Rhizoctonia solani* Kühn are important diseases causing dramatic yield losses in many crops including potato (Stevenson et al. 2001). In the coming years, the phasing out of methyl bromide as a control measure for soilborne pathogens will have a great impact on the accumulation of these pathogens in the soil (Tjamos et al. 2000). Therefore, effective control methods for soilborne pathogens are urgently needed for commercial potato production.

An environmentally friendly alternative to protect roots against fungal and nematode pathogens is bacteria-mediated biological control (Weller 1988; Whipps 1997; Bloemberg & Lugtenberg 2001). One of the difficulties in developing rhizobacteria as an alternative control measure is that many biological control agents are found to be too variable in their performance. Successful biological control not only requires a better understanding of the complex regulation of disease suppression by antagonists in response to environmental factors but also a better picture of the dynamics and composition of plant-associated bacterial communities and what triggers plant colonization. Thus little is known about

microenvironment specificity of antagonistic plant-associated bacteria and their biocontrol potential especially for internal plant colonizers (Krechel et al. 2002).

Endophytic bacteria have been defined by Hallmann et al. (1997) as those bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plant and, additionally, do not visibly harm the plant. It has been suggested that bacteria might interact more closely with the host plant as first expected independent from abiotic conditions in soil and therefore could be efficient biological control agents in sustainable crop production (Sturz et al. 2000).

To examine the impact of the microenvironment on the abundance and diversity of the bacterial communities, especially those from the important functional group of antagonists, isolates from the rhizosphere, phyllosphere, endorhiza and endosphere of field grown potatoes cv. Cilena were analyzed by a multiphasic approach over a two year period. All isolates were screened for antagonism against *V. dahliae* and *R. solani*. Isolates with antagonistic activity obtained a comprehensive phenotypic characterization to find new promising candidates for biological control. The best candidates from the four analyzed microenvironments were assessed as biological control agents.

Material and methods

Isolation of bacteria

On June 12, July 12, August 15 2001 and May 27, June 24, July 17 2002, four potato cv. Cilena plants each were sampled from a potato field in Bonn-Poppelsdorf (Germany). Roots with adhering soil and leaves were collected into sterile bags and then transported to the laboratory. To extract the plant-associated microorganisms, 3 g each of roots and leaves were transferred into a sterile Erlenmeyer flask containing 27 ml of a sterile 0.9 % NaCl solution. The samples were shaken for 10 min. Roots and leaves were then removed for isolation of the endorhiza and endosphere colonizers and the remaining suspension was filtered through Stomacher bags prior to isolation of the rhizosphere and phyllosphere colonizers. For the isolation of endophytic colonizers, roots and leaves were surface sterilized in 2.0 % sodium hypochlorid (NaOCl) supplemented with 0.1 % Tween 20 for 3 min followed by homogenization with mortar and pestle. Before homogenization, the plant material was imprinted on nutrient agar to serve as sterility check. If bacterial growth occurred within 24 hours, samples were discarded. All samples were serially diluted with sterile 0.9 % NaCl and plated onto R2A medium (Difco, Detroit, USA). Plates were incubated for 5 days at 20°C. For each microenvironment 120 colonies with visually different colony morphology were transferred on nutrient agar, purified and stored at -70°C in nutrient broth containing 15 % glycerol until further use.

Identification of plant-associated bacteria and determination of diversity indices

All isolates were identified based on their cellular fatty acids following fatty acid methyl ester gas chromatography (FAME-GC) as described by Sasser (1990) and characterized by the MIDI system (Microbial Identification System, Inc., Newark, USA). Antagonistic isolates were additionally identified by 16S rDNA sequencing aligned with the reference 16S rRNA gene sequence using BLAST algorithm according to Altschul et al. (1996). The general species diversity of the bacterial communities was measured by the Shannon information theory function (Shannon & Weaver 1949). Species richness expressed as the number of species (S) as a function (ratio) of the total number of individuals (N) was determined by the index (d) proposed by Menhinick (1964).

***Screening of bacteria for antagonism towards* Verticillum dahlia *and* Rhizoctonia solani**
All bacterial isolates were screened for their activity towards *Verticillium dahliae* Kleb. and *Rhizoctonia solani* Kühn by a dual culture *in vitro* assay on Waksman agar containing 5 g proteose-peptone (Merck, Darmstadt, Germany), 10 g glucose (Merck), 3 g meat extract (Oxoid, Hampshire, England), 5 g NaCl (Merck), 20 g agar (Difco), filled up to 1 L with distilled water and adjusted to pH 6.8. After 5 days of incubation at 20°C, zones of inhibition were measured according to Berg et al. (2002). The two plant pathogenic fungi, originating from the culture collection of the Department of Microbiology, Rostock University, were routinely grown on Czapek Dox medium (Gibco).

Bacterial characterization based on production of hydrolytic enzymes and secondary metabolites
Production of cell wall-degrading enzymes and secondary metabolites are common mechanisms bacteria use to inhibit fungal growth. For a better characterization of the antagonistic bacterial isolates, their potential to produce hydrolytic enzymes and secondary metabolites was studied. Chitinase activity (β-1,4-glucosamine polymer degradation) and pectinase activity was tested in minimal medium, according to Chernin et al. (1995). Clearing zones were detected 5 days after incubation at 20°C. β-glucanase and cellulase activity was tested using chromogenic azurine-dyed cross-linked (AZCL) and reamazolbrilliant blue R (AZO) substrates (Megazyme, Bray, Ireland), respectively. Formation of blue haloes was recorded until 5 days after incubation. Protease activity indicated by casein degradation was determined from clearing zones in skim milk agar (50 ml sterilized skimmed milk mixed at 55°C with 50 ml 1/5 TSA and 4 % agar) after 5 days of incubation at 20°C. The ability of bacterial isolates to produce indole-3-acetic acid (IAA) was determined using the microplate method developed by Sawar & Kremer (1995). The production of siderophores was tested by growing the bacteria on CAS medium.

Results and discussion

Assessing the best candidates for biological control
A total of 2,648 bacterial isolates were screened for their ability to suppress growth of *V. dahliae* and *R. solani* in an *in vitro* dual culture assay. Altogether, 349 isolates were found to be antagonistic against one or both of these pathogens. They were further characterized based on their production of hydrolytic enzymes and secondary metabolites. In an attempt to better select bacterial isolates with high antagonistic potential an assessment scheme was developed. Therefore, the following points were given for each of the bacterial traits: up to three points were given for antagonistic activity towards *V. dahliae* and *R. solani* and one point each for production of hydrolytic enzymes (chitinases, cellulases, glucanases, pectinases and proteinases), siderophores, and auxin. A total number of 13 points was possible. Promising candidates for biological control were found in all microenvironments. The five best isolates of each microenvironment received between 7 and 12 points (Tab. 1). All twenty isolates showed antagonistic activity towards *R. solani* and 17 isolates against *V. dahliae*. Eighteen out of these 20 antagonistic isolates expressed proteolytic activity and 17 isolates expressed cellulytic activity and siderophore production, whereas only three isolates showed chitinolytic activity.

Table 1. List of the most promising candidates for biological control

Micro-environment	Strain	Species	Antagonistic activity		Cellular production							Asses-ment
					Hydrolytic enzyme					Metabolites		
			V.d.	R.s.	Cellulases	Chitinases	Glucaneses	Pektinases	Proteases	Siderophore	Auxin	
Rhizosphere	1R1-22	*Micrococcus kristinae**	3	3	1	0	0	0	1	1	0	9
	2R2-11	*Arthrobacter oxydans**	3	3	1	0	1	0	1	1	0	10
	2R2-15	*Bacillus licheniformis**	2	2	1	0	1	0	1	1	0	8
	5R3-11	*Streptomyces rochei**	3	3	1	0	1	0	0	0	0	8
	6R4-28	*Pseudomonas chlororaphis**	2	3	0	0	0	0	1	1	1	8
Phyllosphere	1P1-2	*Paenibacillus peoriae*	2	3	1	0	1	1	1	1	0	11
	2P3-18	*Staphylococcus epidermidis*	0	2	1	1	1	1	1	1	0	8
	2P3-25	*Arthrobacter sulfonivorans*	0	2	1	1	1	1	1	1	1	9
	4P2-4	*Bacillus amyloliquefaciens**	2	2	1	0	1	1	1	1	0	9
	5P1-8	n.d.	3	3	1	0	1	1	1	0	0	10
Endorhiza	3Re2-7	*Pseudomonas reactans*	3	3	0	0	0	0	1	1	1	8
	3Re2-26	*Pseudomonas fluorescens*	3	2	0	0	0	0	1	1	1	8
	3Re4-18	*Serratia plymuthica*	3	3	1	1	1	1	1	1	0	12
	3Re4-21	*Pseudomonas putida*	3	2	1	0	0	0	1	1	1	9
	6Re5-7	n.d.	2	3	1	0	0	0	1	1	1	9
Endosphere	1Pe1-14	*Erwinia rhapontici*	3	3	1	0	1	0	1	1	1	11
	1Pe4-13	*Bacillus subtilis*	3	3	1	0	1	0	1	0	0	9
	2Pe3-3	*Bacillus subtilis*	1	2	1	0	1	0	1	1	0	7
	4Pe1-3	*Paenibacillus polymyca**	3	1	1	0	1	1	0	1	0	8
	5Pe4-14	*Bacillus subtilis**	0	2	1	0	1	1	1	1	0	7

Notes: Isolates were identified by partial sequencing 16S rRNA genes or by FAME (marked with *). V.d. = *Verticillium dahliae*, R.s. = *Rhizoctonia solani*, n.d. = not determined

Table 2. Distribution of bacterial species in the different microenvironments of field grown potato plants.

Species	Rhizosphere	Phyllosphere	Endorhiza	Endosphere
Agrobacterium tumefaciens	x		x	x
Arthrobacter globiformis		x		
Arthrobacter ilicis	x			
Arthrobacter oxydans	x			
Bacillus amyloliquefaciens	x	x		
Bacillus aquamarinus				x
Bacillus cereus		x		
Bacillus licheniformis	x		x	
Bacillus mycoides		x		
Bacillus pumilus	x	x	x	x
Bacillus subtilis	x	x	x	x
Bacillus thurginisis	x			
Citrobacter freundii	x			
Curtobacterium flaccumfaciens				x
Enterobacter taylorae			x	
Erwinia amylovora	x			
Erwinia persicinus	x		x	x
Erwinia rhapontici	x			
Lysobacter antibioticus	x			
Micrococcus kristinae	x			
Paenibacillus peoriae		x		
Paenibacillus polymyxa		x		x
Pseudomonas chlororaphis	x	x	x	
Pseudomonas corrugata	x		x	
Pseudomonas fluoresecens	x		x	x
Pseudomonas jessenii		x		
Pseudomonas marginalis	x			
Pseudomonas migulae			x	
Pseudomonas orientalis				x
Pseudomonas putida	x	x	x	
Pseudomonas reactans	x		x	
Pseudomonas savastanoi pv. fraxinus	x		x	
Pseudomonas straminea				x
Pseudomonas syringae			x	
Rhodococcus erythropolis		x		
Serratia plymuthica			x	
Sphingobacterium spiritovorum	x			
Staphylococcus epidermidis	x	x		
Staphylococcus pasteuri	x	x		
Streptomyces halstedii	x			
Variovorax paradoxus	x		x	
Number of species	26	14	16	10

Eighteen out of these 20 isolates could be identified by FAME and or sequencing their 16S rRNA gene resulting in sixteen species belonging to nine genera: *Arthrobacter*, *Bacillus*, *Erwinia*, *Micrococcus*, *Serratia*, *Staphylococcus*, *Streptomyces*, *Paenibacillus*, and *Pseudomonas*. Five isolates having 10 or more points were found: *Arthrobacter oxydans* 2R2-11 (10 points) from the rhizosphere, *Paenibacillus peoriae* 1P1-2 (11 points) and an unidentified isolate 5P1-8 (10 points) from the phyllosphere, *Serratia plymuthica* 3Re4-18 (12 points) from the endorhiza, and *Erwinia rhapontici* 1Pe1-14 (11 points) from the endosphere. These five isolates are considered as the top biocontrol candidates, however, their control potential needs to be confirmed in further tests on host plants at different pathogen pressure and, of cpurse under field conditions.

Distribution of potato-associated antagonistic bacteria in different microenvironments

Of the 349 bacterial antagonists 306 could be identified to the species level (SI > 0.5) by FAME-GC or partial 16S rRNA gene sequencing. Altogether, 41 bacterial species could be identified. The distribution of these species in the different microenvironments is shown in table 2.

The highest number of species with antagonistic properties was isolated from the rhizosphere (26) and endorhiza (16) while 14 and 10 species were found in the phyllosphere and endosphere, respectively. Only two species (*Bacillus pumilus* and *Bacillus subtilis*) were found in all analyzed microenvironments. Microenvironments which showed the most species in common are the endorhiza and the rhizosphere with a total of twelve, half of them are *Pseudomonas* species. Furthermore, rhizosphere and the phyllosphere had six species and the endorhiza and endosphere five species in common. Only four common species were found between the phyllosphere and the endorhiza and between the phyllosphere and the endosphere. Twentysix species (63 %) were exclusively found in one microenvironment. The majority of these species (12) occurred in the rhizosphere, six in the phyllosphere and four each in the endorhiza and endosphere. The diversity was highest in the rhizosphere (2.5) followed by the endosphere (2.0), endorhiza (1.8) and phyllosphere (1.4). This clearly demonstrates the specificity of the bacterial communities in the different microenvironments.

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Multitrophic Interactions in Soil and Integrated Control
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The effect of certain bacteria and fungi on the biology of the root-knot nematode *Meloidogyne* spp.

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Abstract: Plant parasitic nematodes cause great economic losses to agricultural crops worldwide. However, with the increasing environmental pressures, it is necessary to develop alternative methods of control such as biological control, in which microorganisms are selected for their ability to antagonize pathogens. Seven organisms were investigated for their potential as biological control agents against *Meloidogyne* spp.; the fungi *Trihoderma harzianum, Trichoderma viride, Gliocladium virens* and *Fusarium culmorum*; the bacterial *Pseudomonas oryzihabitans* (which is symbiotically associated with the nematode *Steinernema* spp.), *Xenorhabdus nematophilus*; and the obligate parasite bacterium *Pasteuria penetrans*. Studies *in vivo* demonstrated that the application of fungi and *Pseudomonas oryzihabitans* did not prevent both nematode penetration, and development inside the roots. Moreover the interactions between all involved organisms are very complicated. *P. oryzihabitans* may influence nematode development (eggs/egg mass) only in the inter-medium tested inoculum (1000 J_2s/plant). Same results were achieved for *G. virens* and *F. culmorum* at the low (200 J_2s/plant) and high (2000 J_2s/plant) nematode densities. *T. harzianum* seems to have the same effect only in the high nematode density. Clearly only *P. penetrans* revealed significant biocontol activity against the tested root-knot nematode.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) constitute one of the major economic pests, which cause great losses to agricultural crops worldwide (Sharon et al., 2001). Control strategies such as chemical compounds, resistant varieties, crop rotation and many others are available but have arisen a lot of concern. Increasing costs, toxicity to humans, undesirable effects to the biodiversity of natural enemies, limited numbers of resistant varieties are only a few of those concerns. With the increasing environmental awareness, biological methods are being developed as a part of alternative IPM strategies. Due to those problems considerable effort is being directed towards to the use of antagonistic micro-organisms such as the bacterium *Pasteuria penetrans* and *Trichoderma harzianum* which have been described as potential biocontrol agent against several soil borne plant pathogens (Yeoung-Seuk Bae & Knudsen, 2001; Harman et al., 1980, 1984; Wells et al., 1972). This study was conducted to determine the possible potential benefits of those organisms and their interactions with the nematode population.

Materials and methods

In this work, seven organisms were investigated for their potential biocontrol activity against *Meloidogyne* spp. The obligate endospore-forming parasite *Pasteuria penetrans* that has been successfully used for the control of root-knot nematodes (Gowen et al., 1998; Dube & Smart, 1987; Weidelzahl-Fulton et al., 1996; Davies et al., 1988; Channer & Gowen, 1988), the

bacterium *Pseudomonas oryzihabitans* that has been reported to be effective against root-knot nematodes (Andreoglou & Gowen, 2000; Andreoglou et al., 2001; Samaliev, et al., 2000), the bacterium *Xenorhabdus nematophilus* (Paul et al., 1981), the fungus *Fusarium culmorum* and the mycoparasites *Trichoderma harzianum*, *Trichoderma viride* and *Gliocladium virens*.

Fungal antagonists

The *Gliocladium virens* and *Trichoderma viride* isolates were derived from the collection of the Plant Protection Laboratory of the T.E.I. of Larissa, Greece. Both were isolated from greenhouse soil in central Greece (Gravanis & Xifilidou, 2000). The fungus *Fusarium culmorum* provided by Dr. R.T.V. Fox Reading University, and *Trichoderma harzianum* Rifai provided by CABI Bioscience, Egham, were also used in this study. All four isolates were kept on potato dextrose agar (PDA) plates.

Entomopathogenic bacteria

P. oryzihabitans and *X. nematophilus* were isolated from *Galleria mellonella* infected with nematodes using the method described by Akhurst (1983). Pure colonies were multiplied in nutrient broth (3% Nutrient broth No2), the suspension was centrifuged and the bacteria pellets were diluted with sterile tap water. Bacteria concentrations were determined using a spectrophotometer adjusted to the 600nm in wavelength.

Root-Knot Nematodes

Mixed population of the root-knot nematodes *Meloidogyne* spp. was subcultured from a heavy infested commercial tomato greenhouse in Greece and was kept on tomato plants in the University of Reading.

Mass production of second stage juvenile of Meloidogyne spp.

The root-knot nematode population was propagated on tomato plants in the greenhouse. Eggs for inoculation were extracted from infected roots using the method described by Hussey and Barker (1973).

Soil type and inoculation with root-knot nematodes

Pots (750ml) were filled with John Innes No2 loam based compost. Second stage juveniles suspended in water were inoculated onto roots system by adding the required amount of nematode suspension into four holes around a 20 days old tomato plant (c.v. CAL J). Three nematode densities were used; 200 J_2s/plant, 1000 J_2s/plant and 2000 J_2s/plant. Suspensions of both fungal antagonists' spores and entomopathogenic bacterial cells were added into the soil, eight days before tomato plants inoculation with root-knot nematodes. This allowed thorough soil colonization with the bacteria and mycelium to be happened before root-knot nematode infection. However *P. penetrans* applied simultaneously with the nematodes since 5-7 bacterial spores were observed attached onto nematode cuticle. The spore suspension (conidia and bacterial cells), used in this experiment was adjusted at 10^6 spores/ml. The treated plants were kept in a glasshouse at 24-32 °C and were replicated 6 times. Fifty five days after exposure to nematodes, plants were removed, roots were washed, were stained, and fresh and dry stem weights, plant heights, fresh root weights, number of egg masses/g. of root, number of eggs/egg mass, and root-galling indices on a scale of 0-10 were evaluated.

Results and Discussion

Table 1. Effect of certain bacteria and fungi on three densities of *Meloidogyne* spp. in tomato plants

Treatment	2000 J_2s/plant			1000J_2s/plant			200 J_2s/plant		
	Gall index[a,w]	Egg masses/g. of root[b,w]	Eggs/egg mass[c,w]	Gall index[a,w]	Egg masses/g. of root[b,w]	Eggs/egg mass[c,w]	Gall index[a,w]	Egg masses/g. of root[b,w]	Eggs/egg mass[c,w]
P. penetrans	3.3a	2.103a[(5)]	2.358a[(242)]	1.8a	1.000a,b[(1)]	2.318a,b[(213)]	1.8a	1.000a[(1)]	2.329a[(227)]
P.oryzihabitans	5.3b	*4.476b,c[(21)]	♦2.641b[(446)]	5.2b	*4.081a[(18)]	2.266a[(233)]	5.2b	*3.23b,c[(11)]	2.569b[(406)]
X.nematophilus	5.2b	2.814a,b[(9)]	♦2.665b[(479)]	4.2b	2.553b,c[(7)]	2.507b,c[(327)]	3.5ab	*2.57b,c[(10)]	2.474a,b[(316)]
F.culmorum	5.3b	3.332a,b,c[(11)]	2.523b[(363)]	5.2b	3.201b[(11)]	♦2.694c[(504)]	4.6b	1.983a,b[(4)]	2.63b[(446)]
G.virens	5.5b	*5.325c[(30)]	2.528b[(380)]	5.2b	2.563b[(8)]	♦2.682c[(490)]	4.3b	1.848a,b[(4)]	2.595b[(412)]
T.viride	5.2b	*4.37b,c[(20)]	♦2.598b[(412)]	4.8b	*3.969b[(17)]	2.524b,c[(376)]	4.3b	*3.638c[(13)]	2.304a,b[(265)]
T.harzianum	4.8b	*3.67a,b,c[(14)]	2.524b[(380)]	4.8b	*3.891b[(15)]	♦2.573c[(387)]	3.8b	*2.722b,c[(8)]	2.344a,b[(357)]
Root-knot	5.5b	*4.11a,b,c[(21)]	♦2.609b[(410)]	4.6b	*3.807b[(15)]	♦2.52b,c[(349)]	4.3b	*2.724b,c[(8)]	2.503a,b[(337)]

[a]: root-knot nematodes rating chart-Bridge and Page [b]: Numbers were Sqr. transformed [c]: Numbers were log (x10) transformed; ♦: Treatments with the highest production of eggs/egg mass *: Treatments with the highest production of egg masses/g. of root; [w]: Values in columns followed by different letters are significantly different according to Duncan test (P=0.05)

Table 2. Plant height, trop fresh weight and fresh root weight of tomato plants grown in *Meloidogyne* spp. infested soil, treated with certain bacteria and fungi

Treatment	2000 J_2s/plant			1000J_2s/plant			200 J_2s/plant		
	Plant height[a,w]	Top fresh weight[b,c,w]	Fresh root weight[b,c,w]	Plant height[a,w]	Top fresh weight[b,c,w]	Fresh root weight[b,c,w]	Plant height[a,w]	Top fresh weight[b,c,w]	Fresh root weight[b,c,w]
P. penetrans	46.1a	5.075b[(25.9)]	6.477b[(12.5)]	56.6a,b	6.082b[(17.4)]	3.613b[(13.8)]	56.6a,b	6.084b[(37.9)]	5.043c[(26.7)]
P.oryzihabitans	48a,b	4.900b[(24.4)]	5.198a,b[(14)]	58.1b	5.344a,b[(29.4)]	3.653b[(13.9)]	63b	5.665a,b[(32.3)]	3.795a,b,c[(14.6)]
X.nematophilus	49.8a,b	4.740a,b[(23.7)]	5.220a,b[(16.2)]	57a,b	5.127a,b[(26.4)]	3.574b[(12.9)]	62.5b	5.083a,b[(27.2)]	3.762a,b,c[(15.7)]
F.culmorum	55.5a,b	5.358b[(28.8)]	4.719a,b[(12.5)]	57a,b	5.373a,b[(29.1)]	3.627b[(13.9)]	61.3b	5.402a,b[(29.50)]	4.720b,c[(22.5)]
G.virens	55.1a,b	5.164b[(26.7)]	4.485a,b[(10.1)]	56.1a,b	5.599b[(31.5)]	3.950b[(15.9)]	59b	5.702a,b[(33.1)]	4.551b,c[(21.9)]
T.viride	58.5b	5.149b[(26.8)]	4.748a,b[(11.8)]	59.1b	5.459a,b[(30.1)]	3.510b[(12.4)]	59.8b	5.486a,b[(30.3)]	3.348a,b[(12.3)]
T.harzianum	49.5a,b	4.620a,b[(21.6)]	5.110a,b[(14.2)]	51.3a,b	5.045a,b[(25.8)]	2.814a[(8.3)]	53.5a,b	5.098a,b[(26.1)]	3.685a,b,c[(14)]
Root-knot	53.5a	4.044a[(17.3)]	3.823a[(8.6)]	44.6a	4.330a[(19.3)]	2.618a[(7.5)]	47.1a,b	4.514a[(20.9)]	2.554a[(6.7)]

[a]: Plant height in cm [b]: Weights in g. [c]: Numbers were Sqr. Transformed
[w]: Values in columns followed by different letters are significantly different according to Duncan test (P=0.05)

The results suggest that the application of fungi that were tested and the tested bacteria symbiotically associated with entomopathogenic nematodes (*Steinernema* spp.) did not prevent nematode penetration and development inside the roots. Moreover complicated interactions of all involved organisms were observed. This could be explained in part on the ability of those fungi, to proliferate through the soil and the different biotic and abiotic factors including temperature, soil moisture, microclimate, nutrients and many others that may affect the growth and efficacy of biocontrol fungi in soil as others (Sharon et al., 2001; Harman et al., 1984) also reported. Furthermore, it seems that the main antinematode activity caused by *T. harzianum* took place in the soil and not within the roots and is in agreement with Sharon *et al.* results (Sharon et al., 2001). *P. oryzihabitans* may influence nematode development (eggs/egg mass) only in the medium inoculum (1000 J_2s/plant). Similar results were achieved for *G. virens* and *F. culmorum* at the high nematode densities. *T. harzianum* seems to have the same effect at the low nematode density. A possible explanation could be that these fungi and bacteria might produce metabolites toxic to the nematode eggs, being in agreement with others conclusions (Andreoglou & Gowen, 2000; Paul et al., 1981; Samaliev et al., 2000).

Clearly, only *P. penetrans* revealed significant biocontrol activity against the root-knot nematodes possibly due to the application method.

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Resistance induced by soil biocontrol application and soil solarization for the control of foliar pathogens

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Abstract: Induced resistance is recognized as an important mode of disease control with biocontrol agents (BCA). Resistance may be induced locally or systemically, and frequently results in multiple pathogens suppression. A biocontrol agent - *Trichoderma harzianum* isolate T39 controls several foliar pathogens. Modes of *Botrytis cinerea* biocontrol by this BCA were earlier referred to competition and restraining of pathogen pathogenicity enzymes. However, given a spatial separation of the T39 root application from the foliar pathogens infection, the control effect of this BCA and other tested BCAs could be attributed also to induced systemic resistance. Examples of biocontrol of *B. cinerea*, powdery mildews and *Sclerotinia sclerotiorum* are given. *T. harzianum* T39 treatment to soil is associated with a decrease in reactive oxygen species (ROS) in uninfected leaf tissue and in *B. cinerea* induced ROS. Antioxidant enzyme levels were affected as well. Study of *Arabidopsis* hormone mutants, T39 and *B. cinerea* revealed involvement of phytohormones in the triple interaction. Soil solarization result in physical, chemical and microbial changes, including increase in populations of beneficial microoganisms. Similar to *Trichoderma*, solarization of soil results in induced resistance against *B. cinerea* on bean, cucumber and strawberry leaves. Biocontrol of foliar pathogens by *Trichoderma* root treatment or by soil solarization involves not only the beneficial microorganisms and pathogens but also complex plant reactions to both groups of organisms. The indirect plant mediation seems to be also important, in addition to the earlier-emphasized direct pathogen suppression.

Key-words: Biological control, foliar pathogen, soil disinfestation

Introduction

Induced resistance is recognized as an important mode of disease control with biocontrol agents (Elad & Freeman, 2002; Kloepper et al., 1992; Kuc, 1987). Resistance may be induced locally or may be systemic. Induced systemic resistance caused by various micro-organisms can protect plants against soil or foliar pathogens (Paulitz & Matta, 1999). Induced systemic resistance can be achieved by inoculating with a variety of micro-organisms (non-pathogenic micro-organisms, plant growth promoting, and avirulent strains of the pathogen) and can control multiple pathogens. In addition to biotic elicitors of resistance, toxic and non-toxic chemicals, UV, compost and other agents have also been shown to induce resistance (Elad & Freeman, 2002). Raupach & Kloepper (1998) reported that mixtures of plant growth-promoting rhizobacteria (PGPR) caused induced systemic resistance against three foliar and soilborne pathogens of cucumber. Induced resistance to wilt diseases with avirulent fungi has been repeatedly demonstrated under laboratory conditions but under field conditions it was sometimes not effective. Induced resistance results in enhanced and a more rapid production of defense reaction enzymes such as proteases, peroxidases and chitinases, and finally in lignification of the cell walls, limiting pathogen development and spread. Salicylic acid (a

signal molecule in systemic acquired resistance = SAR) produced by *Pseudomonas aeruginosa* induced resistance to *Botrytis cinerea* in bean (De Meyer & Höfte, 1997). Induced systemic resistance is demonstrated by applying a BCA at a location separated from the plant organ that is challenged by a pathogen, whereas the suppression of disease by dead cells of the inducer may demonstrate a local induced resistance.

Induced resistance by Trichoderma spp. and other biocontrol agents

It is now realized that induced resistance is one of the mechanisms frequently involved in biocontrol. Dead cells of antagonistic yeasts and bacteria were found capable of reducing grey mould, caused by *B. cinerea*, similarly to live cultures in some cases (Elad et al., 1994). The application of dead yeast cells was associated with an increase in the indigenous populations of bacteria and yeasts, but the indigenous populations were not high enough to reduce germination of *B. cinerea* conidia and their penetration of the host tissue. The BCAs did not induce resistance when applied at a short distance from the pathogen, and did not produce detectable inhibitory compounds. It was concluded that the activity of the BCAs was associated with their cells or cell walls, and at least part of the activity was not associated with live cells. Similarly, dead cells of *T. harzianum* T39 were also capable of partial control of *B. cinerea* infection (Elad & Kapat, 1999). Thus, locally induced resistance also takes place in the inhibition of grey mould on bean and tomato.

Root inoculation of *T. harzianum* caused increased peroxidase and chitinase activities in leaves of cucumber seedlings, thus indicating induction of defence mechanisms in the plant. (Yedidia et al., 1999). Isolate T39 of *T. harzianum* had a similar induced resistance effect to that of *P. aeruginosa* on bean, challenged by *B. cinerea* (De Meyer et al., 1998). In this work, *T. harzianum* T39 induced plant defence in tomato, lettuce, pepper, bean, and tobacco against *B. cinerea*. The BCA was applied to the soil (in all crops) or to the lower leaves (in beans) whereas the disease was incited on the upper canopy parts of the plants. Disease suppression was up to 100%. The effect of disease suppression declined with time and the decline was faster on the old than on the young leaves. Given the spatial separation of the BCA and the pathogen, this effect was attributed to the induction of systemic resistance by *T. harzianum* T39.

Dead cells of T39 were capable of reducing *B. cinerea* infection on bean, tobacco and pepper (Elad & Kapat, 1999). T39 also controlled powdery mildew on cucumber (Elad et al., 1998) and other powdery mildews. However, *T. harzianum* T39 is not a mycoparasite, nor does it produces inhibitory compounds that can affect such pathogens. Again we could demonstrate the involvement of induced resistance in the disease suppression and relates it to the phenomenon of powdery mildew control. It is possible that the control of soil borne diseases such as *Fusarium oxysporum* f.sp. *dianthi* by T39 can also be related to induced resistance in the root zone. Other modes of action related to *T. harzianum* T39 are competition for nutrients and space and nullifying pathogenicity enzymes of the pathogen. However, this will not be dealt in details in the present manuscript.

The* Sclerotinia sclerotiorum *biocontrol example

Biological control of white mold (caused by *Sclerotinia sclerotiorum*) by isolates of *Trichoderma* spp. isolates was tested on cucumber, lettuce, bean and tomato plants (Elad et al., 2002). Leaves were inoculated by mycelium discs placed on their adaxial (upper) side, whereas *Trichoderma* was applied as a suspension, either to the root zone or to the leaves. Disease control was achieved on the various hosts, but the level of control was affected by factors such as the *Trichoderma* isolate tested, the host species, the plant organ treated, the pathogen isolate, the nature of the biocontrol preparation and the time interval between biocontrol application and pathogen inoculation. An interaction between different factors was

found throughout the experiments. Increased concentration of T39 conidia (from 1 to 5×10^5 ml^{-1}) and application to the leaves and to the roots, as a formulation instead of as naked conidia, increased disease suppression on cucumber, tomato and lettuce. This was true in some of the *Trichoderma* isolates, probably because of their differing requirements. Application of the biocontrol agents 1-5 days before inoculation resulted in better disease suppression by certain isolates. Application to lower leaves resulted in disease control similar to that by soil application in cucumber and bean. Treatment of the abaxial side of the leaves of cucumber and bean resulted in suppression of the adaxial infection also. Dead cells of T39 suppressed white mold as effectively as live cells. The results demonstrate variability in efficacy of biocontrol of *S. sclerotiorum*, among the *Trichoderma* isolates and treated host species Nevertheless, disease reduction of above 70% could be achieved. Apparently, *Trichoderma* activity can be at least partly related to induced resistance.

Involvement of reactive oxygen species and plant hormones in* T. harzianum *mode of action

ROS (as hydrogen peroxide and nitric acid) and the antioxidant enzymes peroxidase, superoxide dismutase and catalase and lipid peroxidtion was tested in leaves of T39-root treated plants. *B. cinerea* infection is associated with increased ROS and consequently antioxidants are elevated. *T. harzianum* T39 treatment to soil is associated with a decrease in ROS in uninfected leaf tissue and in *B. cinerea* affected leaves. T39 in soil is associated with increased antioxidant activity in Botrytis free leaves and with decreased antioxidant activity in the infected leaves. The later is probably due to the reduction in ROS (Lapsker & Elad, 2001). *Arabidopsis* infection by *B. cinerea* was reduced by soil or leaf T39 *T. harzianum* treatments by 40-50%. Preliminary experiments were carried out with *Arabidopsis* mutants related to Abscisic acid (ABA), Gibberellic acid (GA) and ethylene production or perception. *Arabidopsis* mutants that are ABA defficient or insensitive, GA resistant or deficient and ethylene overproducers or insensitive or mutants with reduced production of ethylene were more susceptible to *Botrytis* as compared to the wild type plants. *Trichoderma* was not effective on the ABA and GA mutant plants. T39 was effective in controlling *B. cinerea* on the ethylene mutants. Apparently the ethylene related features are not associated with the biocontrol activity, whereas, GA and ABA features may be involved in T39 incited disease control.

Soil solarization and its mode of action

Soil solarization is a technique for the control of soilborne plant pests (pathogens, weeds and arthropods). It is relatively a new approach for soil disinfestation by means of solar energy (Katan, 1981; Katan & DeVay, 1991, Stapleton & DeVay, 1986). At present, covering (tarping or mulching) the soil with transparent polyethylene, when appropriate climatic conditions prevail, is the only means for capturing solar energy to heat soil under field conditions. The basic process involved in soil solarization is the heating of the soil to relatively mild levels, usually to 36-50^{0}C in the upper 30 cm. Though heat is the major killing agent, there is an accumulating evidence for the involvement of biological processes, which may explain the surprisingly good control sometimes achieved by solarization, even when the temperatures attained were not sufficiently high. Soil solarization aims to eradicate soilborne inocula before planting.

There are certain principles in soil solarization. By solarization, the soil is heated through repeated daily cycles. The maximal temperatures attained decrease with increasing soil depths. Soil has to be kept wet during solarization in order to increase thermal sensitivity of the pathogens and to improve thermal conductivity of the soil. The temperatures attained in

solarized soils are much lower than in steam disinfested ones. Therefore, negative side effects associated with the latter technique are less likely to occur with solarization – though they should not be excluded. Many pathogens, weeds and other pests can be controlled by solarization if climatic conditions are appropriate, namely high levels of temperatures and solar irradiation and low relative humidity. Soil solarization involves physical, chemical (e.g. increase in mineral nutrients) and microbial changes in the soil.

Pathogen control in the solarized soils is attributed to a variety of mechanisms: physical killing by elevated temperatures (thermal killing) and biological processes leading to stimulation of antagonistic activities (DeVay & Katan, 1991). These include induced soil suppressiveness, stimulation of beneficial antagonistic microorganisms, e.g. fluorescent pseudomonads and *Bacillus*, stimulation of antagonistic fungi, the weakening effect and others (Gamliel & Katan, 1991; Greenberger et al., 1987; Stapleton & DeVay, 1985; Tjamos & Paplamotas, 1988). Populations of fluorescent pseudomonads increased considerably in the rhizosphere of plants growing in solarized soil (Gamliel and Katan, 1991) The possible involvement of induced resistance in the host plant (cross protection) although was raised (Katan, 1981), it is only recently that this possibility has been investigated.

Induced resistance by soil solarization

The incidence of some foliar diseases has been surprisingly reduced by solarization in certain cases. This might be attributed to the eradication of primary inoculum, as shown with *Botrytis* (Lopez-Herrera et al., 1999), or to changes in the mineral content of the soil, which may increase plant resistance. However, Gruenzweig et al. (1993) has demonstrated physiological changes in the foliage of tomato plants growing in the solarized soil, including hormonal changes (Gruenzweig et al., 2000) in spite of the fact that only the roots were in contact with the solarized soil. This may indicate that signals were transmitted in the plant upward. Stevens et al. (1992) apparently brought an evidence for a reduction in a foliar disease by solarization, which can be attributed to induced resistance. In a recent study at our laboratory a direct evidence for induced resistance by solarization and by *Trichoderma* against *Botrytis* grey mould in three crops was demonstrated (Table 1). The site of inoculation (leaves) and of induction (roots growing in the solarized soil or treated with *Trichoderma*) are clearly separated, therefore, a systemic effect can be assumed. These findings point to an additional mechanism of disease reduction by solarization, which deserves to be further, investigated.

Conclusion

Induced resistance is evident in the case of soil solarization and *T. harzianum* T39 soil treatment, in addition to other modes of action. There are important questions to be addressed: what are the biotic or abiotic components in the solarized soils, which are responsible for induced resistance? Are the mechanisms of induced resistance and the signals produced and transmitted in the solarization system similar to those in other systems of induced resistance? Can we further enhance the induced resistance generated in the solarized soil? Furthermore, in part of our experiments the control of foliar *B. cinerea* infection achieved by soil solarization followed by *Trichoderma* treatment was more pronounced as compared with each of the soil treatments alone. This opens new horizons for research on integrated disease management.

Table 1. Effect of solarization, *Trichoderma harzianum* (T39) and their combination on incidence of disease caused by *Botrytis cinerea* in five experiments*

Exp No.	Plant	Time after Inoculation (days)	Percent of untreated control		
			Solarization	*Trichoderma harzianum* T39	Solarization + *Trichoderma*
1	Cucumber	3	18	35	6
1	Cucumber	4	25	41	9
2	Cucumber	2	77	62	53
2	Cucumber	3	88	85	81
2	Cucumber	4	102	87	40
3	Strawberry	3	29	45	3
3	Strawberry	4	18	12	1
4	Bean	3	76	70	37
4	Bean	4	116	71	63
5	Bean	3	25	-	-
5	Bean	5	80	-	-

*Plants were grown in solarized soil, or soil was treated with *Trichoderma*, or both. Leaves were inoculated with *B. cinerea*. Disease incidence for each treatment was compared with that in the untreated control, and expresses as % of control (100= no effect; 0= complete reduction of disease). Unpublished results by Neta Okon Levy.

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Survival and activity of the *Ralstonia solanacearum* antagonist *Pseudomonas chlororaphis* 24-4 in the rhizosphere of tomato and its impact on the indigenous bacterial community

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Abstract: Bacterial wilt, caused by *Ralstonia solanacearum*, leads to severe losses in potato cropping. In recent years, biovar 2 (race 3) of the pathogen may have established in European ecosystems. In order to control the pathogen a biological strategy using bacteria with antagonistic characteristics towards *R. solanacearum* was developed. The antagonistic activity of *Pseudomonas chlororaphis* 24-4 to *R. solanacearum* biovar 2 was tested with tomato grown in pots under greenhouse conditions. The fate of the pathogen and the antagonist as well as bacterial community shifts was followed by plating on selective media and by analysis of directly extracted nucleic acids. To provide information on the metabolically active fraction of rhizobacteria a bromodeoxyuridine (BrdU) method was established and optimised. Changes in the relative abundance of bacterial rhizosphere populations during the greenhouse experiment were analysed by PCR-DGGE analysis of 16S rDNA fragments amplified from total community DNA and from DNA extracted after BrdU incorporation. Application of the antagonistic strain *Pseudomonas chlororaphis* 24-4 led to a clear suppression of the pathogen and reduced wilting symptoms. The DGGE profiles confirmed the decreased abundance of *R. solanacearum* in the rhizosphere of tomato plants inoculated with the antagonist determined by selective plating. Comparison of DGGE profiles of 16S rDNA fragments amplified from directly extracted community DNA and BrdU-DNA indicated that *R. solanacearum* was metabolically active in the rhizosphere of plants inoculated with the antagonist as well as in the rhizosphere of non-inoculated plants 21 days after inoculation. Also *P. chlororaphis* 24-4 which was efficiently colonising the tomato rhizosphere was metabolically active throughout the experiment.

Key words: bacterial wilt, antagonists, biological control, DGGE, BrdU

Introduction

Ralstonia solanacearum biovar 2 (race 3), the causative agent of wilting disease in a range of host plants such as potato, tomato and eggplant (Hayward, 1991), can cause devastating problems in agriculture. This organism belongs to the indigenous microflora in tropical regions (Hayward, 1991). However, in recent years, the bacterium increasingly occurred in more temperate climates, e.g., in several West European countries, leading to major losses to commercial potato producers. Numerous outbreaks of biovar 2 strains have been observed in the last ten years in Europe where it is considered as a quarantine bacterium (Wenneker et al., 1999, van Elsas et al., 2000). Due to the utmost importance of the pathogen several studies were initiated to investigate ecology, survival, and establishment of the organism (Granada & Sequeira 1983, Nesmith & Jenkins 1983, Elphinstone 1996, van Elsas et al. 2001).

In order to suppress the disease different soil management strategies (addition of compost and manure, solarisation) were tested (Schönfeld et al. 2003). In addition soils were found that show an enhanced suppression of the disease (Ho et al. 1998). Suppressiveness might be caused by naturally occurring rhizobacteria with antagonistic potential (Ho et al. 1998) and

could be enhanced due to an increased abundance and activity of antagonistic microbial populations induced by adding compost (Schönfeld et al. 2003). The antagonistic effect might be due to different mechanisms: antagonistic bacteria may reduce the incidence of bacterial wilt directly, e.g. by antibiosis (Weller 1998), or they are good rhizosphere colonisers and therefore competitors to *R. solanacearum* at the root surface (Misaghi et al. 1992 & van Overbeek et al. 2002) or they induce systemic resistance (ISR) in the plant (van Loon et al. 1998).

This study should contribute to develop a biological control strategy towards *R. solanacearum* using rhizobacteria with antagonistic properties. The bacterial strain used in this experiment was isolated by Schönfeld et al. (2003, submitted). Briefly, dilutions of soil samples were plated on a poor nutrient agar medium in which *R. solanacearum* was embedded. Colonies surrounded by a clear inhibition zone were picked, first tested *in vitro*, and a collection of them later *ad planta* for their antagonistic capacity. Especially strain *Pseudomonas chlororaphis* 24-4 showed a clear suppression of the pathogen, reduced wilting symptoms, and a good rhizosphere competence. The antagonistic activity of this strain was tested in several greenhouse experiments on tomato with *R. solanacearum* B3B. Plants inoculated with the antagonist showed less wilting symptoms than plants non-inoculated with strain 24-4 (Schönfeld et al. 2003, submitted).

These experiments were repeated now using the more aggressive strain *R. solanacearum* 1609 to confirm the antagonistic effect of *P. chlororaphis* 24-4. The fate of the pathogen and the antagonist were followed cultivation-based by plating on selective media and cultivation-independent by the analysis of directly extracted DNA by PCR-DGGE. To provide information on the metabolically active fraction of rhizobacteria the BrdU method described by Borneman (1999) was used.

Material and methods

Bacteria and growth conditions

R. solanacearum strain 1609, belonging to race 3 (biovar2) was used to infect greenhouse-grown tomato plants (*Lycopersicon esculentum* Mill. cv "Moneymaker"). Strain 1609 was grown on YPGA (yeast extract-peptone-glucose-agar) at 28°C for five days to enrich cell mass for inoculation. The strain was chosen due to its stable aggressiveness towards tomato, potato and egg plant.

Pseudomonas chlororaphis strain 24-4 was used to test its antagonistic potential against *R. solanacearum*. This strain showed a good rhizosphere competence, was able to inhibit the growth of *R. solanacearum* strains 1609 and B3B *in vitro* and suppressed the disease symptoms *ad planta* (Schönfeld et al., in prep.). A spontaneous rifampicin-resistant mutant (50 µg of rifampicin per liter of medium) with no reduction in fitness and rhizosphere competence was chosen for the greenhouse experiments. To produce sufficient cell mass for the greenhouse experiments, the strain was grown on King's B agar medium at 28 °C for 48 h (Merck KGA, Darmstadt, Germany).

Tomato plants (*Lycopersicon esculentum* Mill.) of the susceptible cultivar "Moneymaker" were cultivated in a greenhouse chamber at 21°C in pots containing 150 g of a commercial potting substrate (Clay substrate of Klasmann-Deilmann GmbH, Geeste-Gross Hesepe, Germany) until the three-leaf stage. For the experiments this soil was mixed with sand in a 4:1 ratio.

Inoculation

Suspensions of *R. solanacearum* strain 1609 and *P. chlororaphis* strain 24-4 were prepared by rinsing agar plates (YPGA for *R. solanacearum* B3B and KB for *P. chlororaphis 24-4)* with 8 ml of sterile 0.85 % NaCl solution. Cell densities were adjusted by measuring the optical density in a spectro-photometer and subsequently diluting with sterile water.

For inoculation, the cell density of strain 24-4 was adjusted to approximately 10^{10} cells/ml of suspension. The tomato plants were dipped in the suspension for 5 - 10 minutes and then planted in the potting soil.

The cell number of the pathogen was adjusted to an inoculum size of 3 x 10^6 cells per g of soil. The cell suspension was used to water the plants three times at day 0, 2 and 4. Plants were kept at 28 °C and natural light conditions (time 12 h light/dark) in the greenhouse.

Experimental setup

For the experiment, the test plants were differently treated as follows. I: no inoculation (control, C), II: inoculation with *R. solanacearum* only (Ral), and III: inoculation with *R. solanacearum* and the potential antagonist (Ral + 24-4). To assess a potential plant growth promoting effect of the treatment with strain *P. chlororaphis* 24-4, inoculation with strain 24-4 only (treatment IV: 24-4) was included. Each treatment was prepared twice in parallel and consisted of two sets of 36 plants each. The plants, one per pot, were grown in a greenhouse chamber at 28°C.

The severity of disease symptoms was evaluated over a period of 35 days without destructively taking samples with the first set of plants. In addition, the development of cell numbers of the pathogen, the potential antagonist and the total number of culturable bacteria and the molecular analysis were assessed following destructive sampling of tomato plants of the second set of plants.

Evaluation of disease symptoms

Disease severity was evaluated visually and scored using an index with a range of 0 to 5 as follows. 0: healthy-looking plant; 1: one leaf beginning to wilt; 2: a second leaf wilted; 3: either a third leaf wilted or one or more complete branches wilted; 4: a completely wilted but still green plant; and 5: a dead plant with stem and leaves dry according to Schönfeld et al. (2003, submitted). The plants were observed over a period of 35 days.

Sampling, extraction of bacterial cells from roots and plating

Rhizosphere samples consisted of 3 g (wet weight) of roots with adhering soil pooled from three plants. For each treatment, three such composite samples were taken. All treatments were sampled at the same time points at days 1, 7, 14 and 21. Three grams plant roots with firmly adhering soil were transferred into sterile plastic bags resuspended in 9 ml of distilled water and treated in a Stomacher 400 blender (Seward) at high speed for 1 min. After this treatment the supernatant without roots was collected in a Sorvall tube and the remaining soil or roots were resuspended in 9 ml of distilled water, followed by Stomacher blending. This step was repeated once. The supernatants of the three steps were combined. One ml was used for serial dilution and plating, the rest was centrifuged at high speed (20,000 x *g*) for 30 min to collect the microbial pellet. The pellet was resuspended in 1-2 ml sterile water and transferred into one to two Eppendorf tubes with a pipette and centrifuged at 10,000 x *g* for 20 min. The resulting pellet was kept for DNA extraction at -20 °C.

Counts of colony forming units (cfu) of aerobe heterotrophic bacteria were determined on R2A medium (Difco Laboratories, Detroit, Michigan), supplemented with 100 mg/l of cycloheximide (Sigma, Steinheim, Germany), after 5 days of incubation at 28°C.

Culturable *R. solanacearum* populations were enumerated by plating onto the semi-selective medium SMSA (culturable counts of presumptive *R. solanacearum*; (Engelbrecht 1994, Elphinstone 1996) after 5 days of incubation at 28 °C. Colony numbers of the rifampicin resistant mutant of the potential antagonist *P. chlororaphis* 24-4 were counted on King-Agar B, supplemented with 50 mg/l rifampicin after 2 days of incubation at 28 °C.

DNA extraction and PCR-DGGE

Total community DNA was extracted from 0.25 to 0.5 g of microbial pellet with the UltraClean™ Soil DNA Isolation Kit (MoBio Lab., Solana Beach, USA). DNA extraction was done according to the manufacturer's protocol including a bead beating step (2 x 30 sec at 4,000 rpm) with an MSK cell homogeniser (B. Braun Diessel Biotech, Melsungen, Germany) for mechanical cell lysis.

PCR and DGGE analyses were performed following the protocols given by Heuer et al. (2001), and Gomes et al. (2001). Eubacterial and beta-proteobacteria-specific PCR and *Pseudomonas* selective PCR was done according to Milling et al. (2003, submitted). Bacterial community fingerprints were analysed by the GelCompar 4.0 program (Applied Maths, Ghent, Belgium) according to Rademaker et al. (1998) with a slight modification of normalisation settings (Smalla et al., 2001).

BrdU immunocapture

To provide information on the metabolically active bacterial fraction the BrdU method described by Urbach et al. (1999) and Borneman (1999) was adapted for the study of rhizosphere bacteria. Briefly, the rhizosphere samples (microbial pellets) were incubated with 5 µM BrdU at room temperature in the dark for 48 h. DNA was extracted using the BIO101 soil DNA extraction kit (Q-BIOgene, Carlsbad, USA) followed by an immunocapture step with anti-BrdU-coated magnetic beads and recovery of the DNA after immunoprecipitation as described by Borneman (1999). The 16S rDNA fragment spanning the variable regions V6-V8 (*E. coli:* 948 -1401) (Brosius et al. 1981) was amplified by PCR from the DNA recovered directly or after group-specific PCR and analysed by DGGE as described above.

Results and discussion

Evaluation of disease symptoms

Plants were observed over a period of 35 days after inoculation and wilting symptoms were scored using a disease index (0-5). All non-inoculated control plants (I), respectively, plants inoculated only with the antagonist (IV) were healthy over the whole period of the experiment. Plants only inoculated with *R. solanacearum* 1609 (II) showed the first wilting symptoms after 8 days, and after 35 days 95 % of the plants (34 plants) were completely wilted. In contrast, the inoculation with the antagonist (III) led to a clearly retarded occurrence of disease symptoms, because the first wilting symptoms became apparent only after 10 days and the first dead plants were detected after 14 days. At the end of the sampling period after 35 days only 50 % of the plants were completely wilted, 2 % of the plants showed first wilting symptoms. However, the remaining plants (48 %) were healthy.

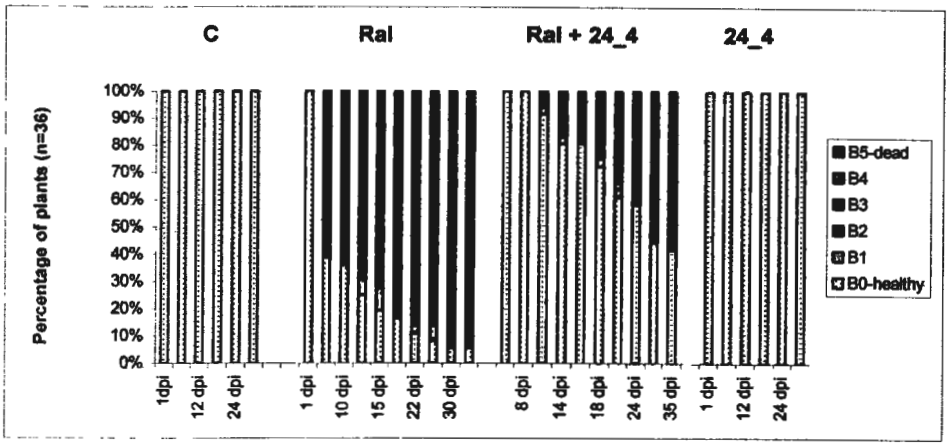

Figure 1. Evaluation of severity of disease symptoms over 35 days using a disease index system with 6 steps (0-5)

In the previously performed experiment using *R. solanacearum* B3B the wilting process started as well after 7-8 days. At the end of the sampling period after 28 days, 78% (28 plants) of the plants which were only inoculated with the pathogen were dead, and another 13% of plants showed severe wilting symptoms. In contrast, plants additionally inoculated with the antagonist were almost completely healthy, only one plant was dead. All non-inoculated control plants, respectively, plants inoculated only with the antagonist were healthy over the whole period of the experiment (Schönfeld et al. 2003, submitted).

Bacterial counts

During the first week the bacterial titer of *R. solanacearum* 1609 was enormously increased due to repeated watering with the pathogen within the first 6 days. Counts of *R. solanacearum* on SMSA medium displayed rising *Ralstonia*-cfu numbers by more than 3 orders of magnitude at plant roots not inoculated with the antagonist (II), respectively, 1.5 orders of magnitude at plant roots inoculated with the antagonist (III) within the first week. These dynamics continued until the end of the experiment. After 21 days the average cfu per gram rhizosphere of *R. solanacearum* 1609 reached log 9.9 at plants not inoculated with the antagonist (II), respectively, log 7.8 at plants inoculated with the antagonist (III). The cfu numbers of *R. solanacearum* were significantly different when plants with and without inoculation by the antagonist were compared. A significantly lower titer of *R. solanacearum* was detected for tomato plants treated with the *P. chlororaphis* 24-4 at the end of the experiment (Fig. 2). The bacterial titer of *P. chlororaphis* 24-4 decreased from log 10 (IV - 24-4), respectively, log 9 (III - Ral + 24-4) by 2, respectively, by 1.5 orders of magnitude over the experimental period. After two weeks *Pseudomonas* cell numbers were stable and resulted in an average of about 10^6 cfu per gram rhizosphere. The presence of the pathogen did not affect the titer of the antagonist (Fig.2).

These cultivation-based results obtained in the presented study were comparable with the experimental results obtained by Schönfeld et al. (2003, submitted) using *R. solanacearum* B3B. A significantly lower titer of *R. solanacearum* B3B was detected for tomato plants treated with the *P. chlororaphis* 24-4 already after 7 days post planting. The antagonistic

strain 24-4 showed a good rhizophere competence, and a stable titer of 10^7 cfu per gram rhizosphere could be established.

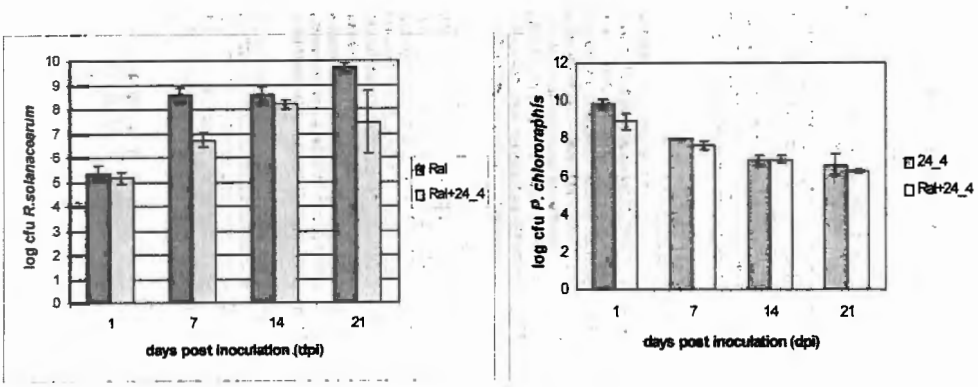

Figure 2. Bacterial counts of *R. solanacearum* 1609 on SMSA medium (left) and *P. chlororaphis* 24-4 on King's B agar medium supplemented with 50 mg/l rifampicin (right)

Detection of* R. solanacearum 1609 *and* P. chlororaphis 24-4 *in the rhizosphere of tomato plants by PCR-DGGE

Recent results indicated that parts of a *R. solanacearum* cell population are capable to convert to VBNC -viable but nonculturable- forms (van Elsas, 2000, Grey and Steck, 2001, van Elsas 2002, van Overbeek, 2002). The observation of these VBNC cells, but also the appearance of symptomless infections of the pathogen indicate the limitation of cultivation-based methods. Hence, the fate of the pathogen and of the antagonist as well as bacterial community shifts were followed by the analysis of directly extracted DNA (direct DNA) and PCR-DGGE of 16S rDNA fragments. In addition, the development of the metabolically active bacterial community during the greenhouse experiment was followed by PCR-DGGE analysis of 16S rDNA fragments amplified from total community DNA extracts incorporating BrdU (BrdU-DNA). DGGE profiles of non-BrdU incorporating and BrdU incorporating bacterial communities extracted from rhizosphere samples taken 1, 7, 14 and 21 days post planting were compared.

PCR-DGGE analysis of rhizopshere DNA extracts from tomato plants revealed complex patterns of eubacterial communities for all four treatments. While a band with an electrophoretic mobility similar to that of *R. solanacearum* 1609 was clearly observed for samples from plants which were not inoculated with the antagonist in both the direct DNA and the BrdU-DNA, this band was only faint or not visible in the eubacterial community patterns of direct and BrdU-DNA from plants inoculated with the antagonist. After 21 days the band corresponding to *R. solanacearum* could be only detected in the DGGE profiles in one of three replicates of plants inoculated with the antagonist (Fig. 3). *R. solanacearum* 1609 belonged to the dominant populations in the rhizosphere of plants not inoculated with the antagonist, and the intensity of this band was increased during the experiment correlating with the bacterial counts obtained (Fig. 4). The band comigrating with those of the antagonist *P. chlororaphis* 24-4 was detected in the eubacterial community patterns only in the first two weeks of the experiment as expected from the bacterial counts. Cluster analysis revealed a clear influence of the different treatments on the rhizosphere community structure in DGGE

profiles of 16S rDNA fragments 21 days after inoculation compared to the control plants (Fig. 3). In order to increase the sensitivity while reducing the complexity of DGGE patterns, primers specific for β-Proteobacteria to detect *R. solanacearum*, respectively, Pseudomonads to detect *P. chlororaphis* were used to analyse rhizosphere communities. Bands comigrating with that of both the pathogen and the antagonist were observed in the DGGE profiles of direct DNA and BrdU-DNA after group-specific PCR (data not shown). These results confirmed that both the pathogen and the antagonist were present in the respective treatment and indicated metabolical activity of R. *solanacearum* 1609 as well as of *P. chlororaphis* 24-4 over the whole time of the experiment.

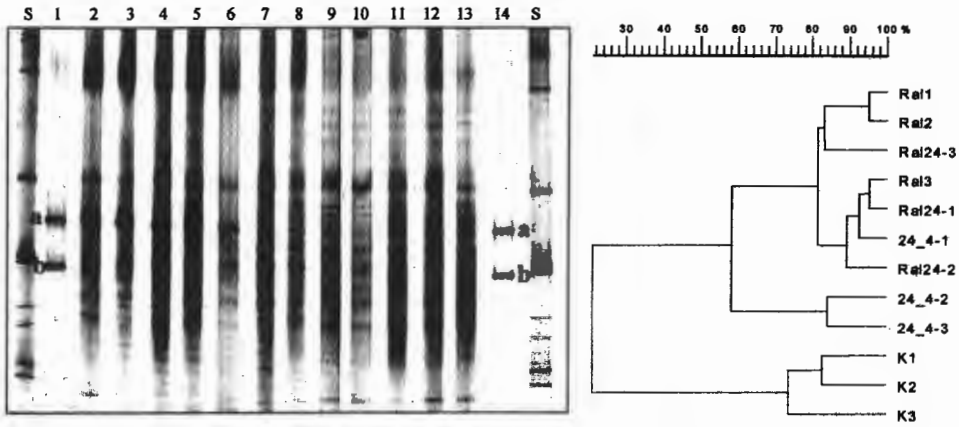

Figure 3. DGGE profiles of Eubacteria generated from 16S rDNA fragments (*E. coli*: 968-1401) 21 days post planting and dendrogram generated by cluster analysis using Gelcompare 4.0, directly extracted rhizopshere DNA. S- standard (Heuer et al. 2001), 1,14 – a: R. *solanacearum* 1609, b: *P. chlororaphis* 24-4, 2-4 treatment II (Ral), 5-7 treatment III (Ral + 24-4), 8-10 treatment IV (24-4), treatment I (control)

There was a clear difference between the DGGE profiles of plants with and without BrdU incubation. The intensity of at least one band increased drastically in the profiles of all DNA incorporated BrdU compared to the profiles generated from directly extracted DNA (Fig. 4). The results obtained in the presented study confirmed the experimental data from Schönfeld et al. (2003, submitted) using *R. solanacearum* B3B. However, differences concerning the metabolically activity of the pathogen were detected. In the latter trial, the β-proteobacterial pattern generated from BrdU-DNA indicated that *R. solanacearum* B3B had a lower metabolic activity in the rhizosphere of plants inoculated with the antagonist compared to plants non-inoculated with the antagonist.

PCR-DGGE was a suitable tool to follow the fate and survival of the pathogen as well of the antagonist. The influence of the different treatments in the indigenous microflora was revealed. To provide information on the metabolically active bacterial fraction the bromo deoxyuridine (BrdU) method (Urbach et al. 1999, Borneman 1999) was used. However, a further evaluation of the method is necessary regarding the efficiency of BrdU uptake and incorporation into genomic DNA of different species to avoid misinterpretation of the DGGE profiles of metabolically active bacteria.

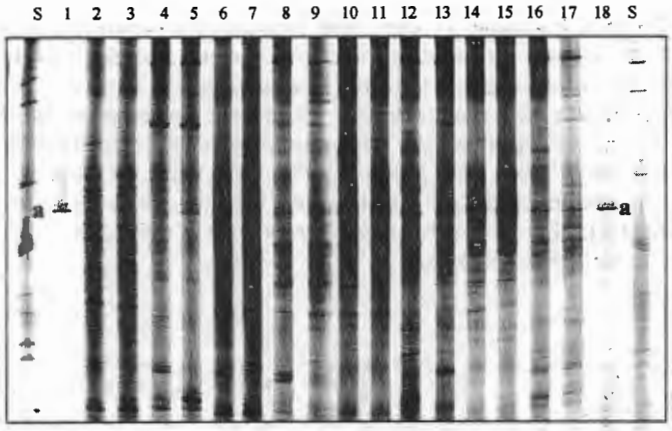

Figure 4. DGGE profiles of Eubacteria generated from 16S rDNA fragments (*E. coli*: 968-1401) 21 days post planting , treatment II (Ral), Comparison of directly extracted rhizosphere DNA and BrdU-DNA. S- standard (Heuer et al. 2001), 1,18 – a: R. *solanacearum* 1609, 2,3 1 dpi – DNA, 4,5 1 dpi – BrdU-DNA, 6,7 7 dpi – DNA, 8,9 7 dpi – BrdU-DNA, 10,11 14 dpi – DNA, 12,13 14 dpi - BrdU-DNA, 14,15 21 dpi - DNA, 16,17 21 dpi – BrdU-DNA (dpi – days post inoculation)

Conclusions

The successful application of the antagonistic strain *Pseudomonas chlororaphis* 24-4 to control the plant pathogen *Ralstonia solanacearum* was shown under greenhouse conditions. Due to application of the antagonist, the outbreak of the disease was delayed and a clear reduction of the disease's severity was observed. However, even in the stem and leaves of healthy looking tomato plants *Ralstonia* was detected by bacterial counts on SMSA (data not shown). The invasion of the plant by the pathogen was hampered by application of strain 24-4 but not abolished. The expression of virulence genes is dependent on the cell density. The pathogenicity system of *R. solanacearum* is only activated at cell densities above 10^7 cells/ml (Schell, 2000). This might explain the protection of plants against bacterial wilt by the treatment with the antagonist.

Latent infections of plants with *R. solanacearum* can be caused by a *Ralstonia* titer below the cell densities required to express virulence genes and/or by variation of pathogenicity. Furthermore, it was shown that even a highly virulent *R. solanacearum* isolate can become non-pathogenic under certain conditions. In this case, *R. solanacearum* is established in the plant, but no wilting symptoms become apparent. However, the non-pathogenic form of the pathogen can convert to a pathogenic form (van Overbeek et al., 2002).

Numerous outbreaks of biovar 2 strains are being reported in the last ten years in Europe and the importance of bacterial wilt is increasing. Europe-wide clear and very strict rules exist to prevent the spreading of the quarantine organism *R. solanacearum*. Approved phytosanitary management and control strategies must guarantee a zero bacterial titer of the pathogen in the plant. Biocontrol measures cannot achieve this strict demand at the moment, although there is no doubt that potato producers are interested in the application of alternative

control strategies. However, the use of biocontrol strategies as demonstrated in this study might be helpful to control the disease in the tropics, where *R. solanacearum* is endemic.

Acknowledgements

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Biological variation in *Verticillium chlamydosporium* isolated from different nematode hosts

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Abstract: *Verticillium chlamydosporium* is a potential biological control agent for root knot and cyst nematodes. It was isolated from infected eggs of *Meloidogyne incognita* and *Globodera rostochiensis*. It was found that an isolate from *M. incognita*, Vc 10, had a higher radial growth rate and metabolic activity than an isolate from *G. rostochiensis*, Vc Jersey. However Vc Jersey showed greater proteolytic activity and showed higher activity of the enzyme VCP1. Purification of VCP1 from both isolates revealed two activity peaks in Vc Jersey, at pH 10 and 10.2 but one in Vc 10, at pH 10.2. SDS-PAGE showed a band doublet in Vc Jersey at pH 10 that did not occur in Vc 10. These data indicate that there is variation between isolates of *V. chlamydosporium* that relates to its potential virulence against nematode eggs.

Key Words: *Verticillium chlamydosporium*, VCP1, biological control.

Introduction

Root knot (*Meloidogyne* spp) and cyst (*Globodera* spp and *Heterodera* spp) are significant pests of crops causing an estimated US$ 70 billion of damage each year (Sasser & Freckman, 1987). On high value crops these pests have been controlled by the application of chemical nematicides but their usage is to be reduced because of the effects of some of products on the environment (Thomas, 1996). Nematodes are subject to parasitism by a variety of organisms including bacteria and fungi. The bacterium *Pasteuria penetrans* and the fungi *Paecilomyces lilacinus* and *Verticillium chlamydosporium* have been tested for their ability to act as biological control agents of these nematodes (de Leij *et al.*, 1992 & 1993).

Alkaline serine proteases have been implicated in the infection processes of many invertebrate mycopathogens. Biological control agents such as *Metarhizium anisopliae* (St. Leger *et al.*, 1987) and *Beauvaria bassiana* (Bidochka & Khachatourians, 1990) secrete alkaline serine proteases to assist in penetration of the insect cuticle. Similar proteases have been identified in the nematophagous fungi *Arthrobotrys oligospora* (Tunlid *et al.*, 1994), *P. lilacinus* (Bonants *et al.*, 1995) and *V. suchlasporium* (Lopez-Llorca 1990).

An alkaline serine protease VCP1 was identified from *V. chlamydosporium* (Segers *et al.*, 1994). Assays with the enzyme indicated that it was involved in the digestion of the nematode eggshell (Segers *et al.*, 1994).

Materials and Methods

Fungal cultures
Isolates of *V. chlamydosporium* were obtained from the Rothamsted culture collection and maintained on corn meal agar (Oxoid) in darkness at 28 °C. Liquid cultures were made by inoculating Czapek Dox broth (sucrose 30 g l^{-1}, sodium nitrate 3 g l^{-1}, di-potassium hydrogen

phosphate 1 g l^{-1}, magnesium sulphate 0.5 g l^{-1}, potassium chloride 0.5 g l^{-1} and yeast extract 0.5 g l^{-1}) with 5mm plugs from stock cultures and incubated with shaking, 110 rpm, at 28 °C.

Measurement of growth, protease production and metabolism

The growth of fungal isolates was determined by measuring the radial growth rate of colonies on chocolate agar plates (Becton Dickinson). The plates were inoculated by placing a 5 mm plug from stock cultures in the centre of the fresh plate. Protease production was measured as the diameter of the zone of clearing around the colony perimeter on chocolate agar (Chalupova & Lenhart, 1984). Metabolic activity was measured by inoculating the wells of microtitre plates containing 150 µl of Czapek Dox broth containing 0.002 % tetrazolium violet with spore suspensions of 5000 spores ml $^{-1}$. Metabolic activity was measured as the change in absorbance of the cultures at 590 nm (Atkins, 2000).

Purification of VCP1 and isoelectric focusing

VCP1 was purified by precipitating proteins from *V. chlamydosporium* culture filtrate with solid ammonium sulphate to 80 % saturation. The sample was dialysed and applied to the Rotofor system (BioRad) for preparative isoelectric focusing, IEF, (Segers *et al.*, 1994). Enzyme activity was measured as the release of p-nitroanilide from the chromogenic substrate N-Succ-$(Ala)_2$-Pro-Phe-p-nitroanilide (Sigma) at 410 nm. Purified enzyme was visualised on SDS-PAGE using Laemmli buffers (Sambrook *et al.*, 1989) and stained with Sypro Ruby protein gel stain (Molecular Probes).

Results

Isolate Vc 10 (from *M. incognita*) had a higher growth rate than isolate Vc Jersey (from *G. rostochiensis*) on chocolate agar (Figure 1). Vc 10 had a slightly higher metabolic activity in Czapek Dox broth than Vc Jersey (Figure 2). Vc Jersey caused more clearing of chocolate agar than Vc 10 indicating a greater production of proteases (Figure 1).

IEF showed that there were two activity peaks in culture filtrate from Vc Jersey and only one from Vc 10 (Figure 3). The peaks in Vc Jersey occur at pH 10 and pH 10.2. The peak in Vc 10 occurs at pH 10.2. The fractions with the highest activity from both Vc 10 and Vc Jersey contained a single band but the second peak from Vc Jersey contained two bands. The size of the single band from Vc Jersey was slightly less than that of Vc 10 but was in the 30 kDa range characteristic of fungal serine proteases (Figure 4).

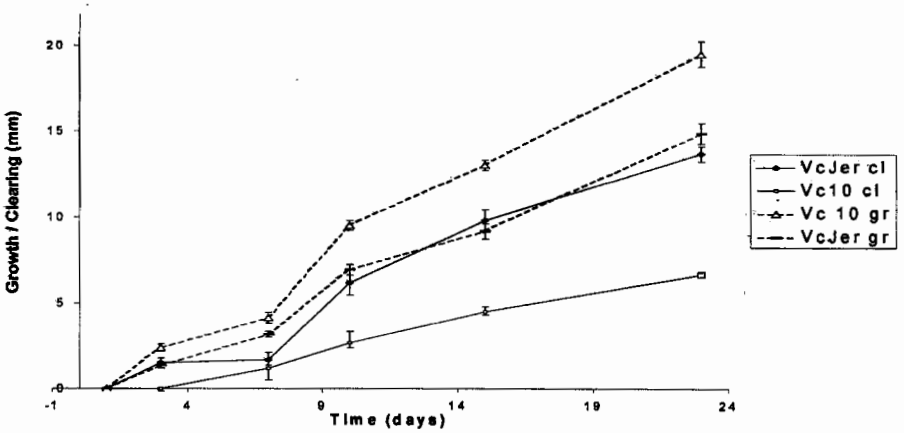

Figure 1. Radial Growth (gr, dashed lines) of *V. chlamydosporium* isolates on chocolate agar and clearing (cl, solid lines) caused by proteolytic activity.

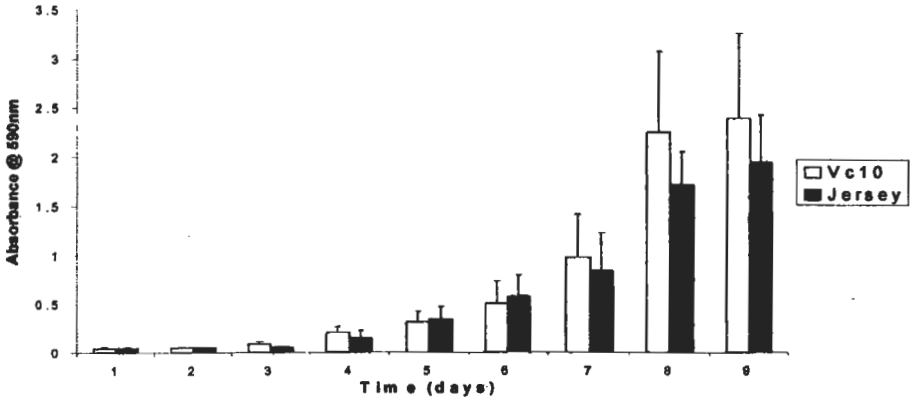

Figure 2. Metabolic activity of isolates of *V. chlamydosporium* measured as the change in absorbance of tetrazolium at 590 nm.

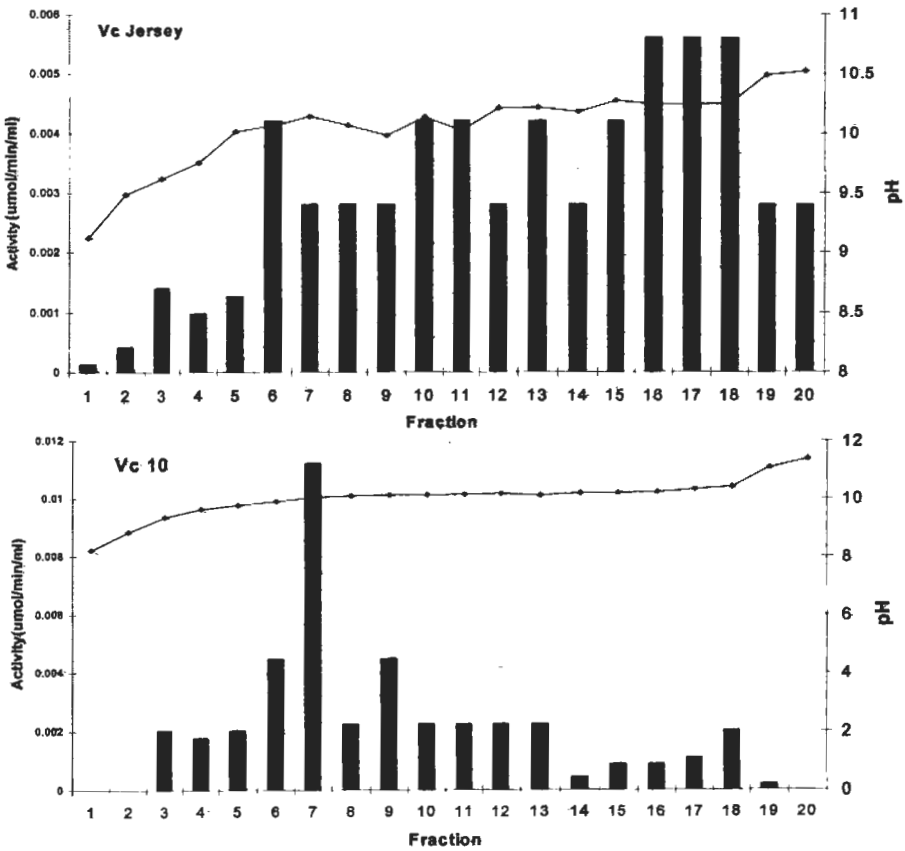

Figure 3. Enzyme activity and pH of fractions obtained by IEF of culture filtrates from *V. chlamydosporium* isolates Vc Jersey (top) and Vc 10 (bottom).

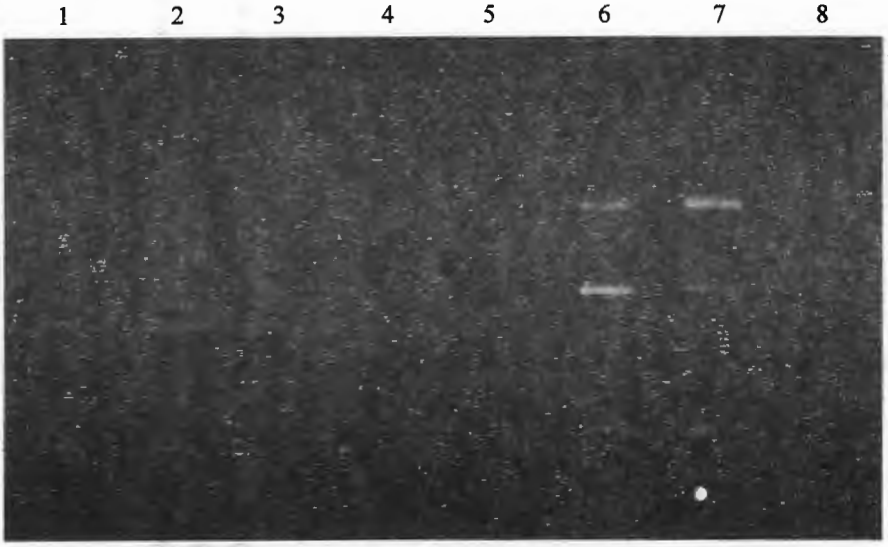

Figure 4. SDS-PAGE gel showing samples from the purification of VCP1 from culture filtrates from *V. chlamydosporium* isolates Vc Jersey and Vc 10. The number of bands reduces as the purification process proceeds. The bands corresponding to VCP1 are indicated by arrows. The band doublet that occurs in Vc Jersey is shown in lane 3.
Lane order: 1, concentrated culture filtrate; 2, dialysed culture filtrate; 3, IEF fraction at pH 10; 4, IEF fraction at pH 10.2; 5, IEF fraction at pH 10.2; 6, dialysed culture filtrate; 7, concentrated culture filtrate; 8, culture filtrate. Lanes 1 – 4 are Vc Jersey and lanes 5 – 8 are Vc 10.

Discussion

In this report *V. chlamydosporium* isolate Vc 10 (*M. incognita*) had a higher radial growth rate and higher metabolic activity in Czapek Dox broth than Vc Jersey (*G. rostochiensis*). The slower radial spread of Vc Jersey may be due to its capacity to utilise a greater proportion of the substrate due to its greater proteolytic potential, this was reflected in the denser colonies formed by Vc Jersey.

The ability to clear chocolate agar was correlated to nematicidal potential in mutants of *V. chlamydosporium* (Chalupova & Lenhart, 1984). These data indicate that Vc Jersey might be a more efficient egg parasite than Vc 10 by virtue of its superior protease production. SDS-PAGE analysis of VCP1 from both isolates showed that the enzyme from Vc 10 was slightly larger than that from Vc Jersey, this may be attributable to charge differences between them, indicating potential intraspecific variation. RFLP analysis of different isolates of *V. chlamydosporium* had suggested possible intraspecific variation between isolates (Segers *et al.*, 1999). These differences may affect nematode host selection since VCP1 is important for host infection (Segers *et al.*, 1994) and it has been shown that some isolates vary in their ability to infect particular hosts (Crump & Irving, 1992).

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Dissecting the tri-trophic interaction between *Pochonia chlamydosporia*, root-knot nematodes and their plant hosts.

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Abstract: The nematophagous fungus *Pochonia chlamydosporia* is a potential biological control agent for root-knot and cyst nematode species. The fungus switches from a saprophytic to a parasitic phase and infects nematode eggs. An important factor in this process is the production of the protease VCP1, which may act as an indicator of virulence. The plant or nematode derived factors that affect its regulation, *in situ*, need to be elucidated. The fungus, plant and nematode form a tri-trophic interaction but the factors that control these interactions and changes in the trophic phase of the fungus are unknown. Plants have been grown in axenic culture and root exudates collected from these plants to examine their effect on the fungus. It was shown that cabbage root exudates supported sparse growth of the fungus compared to tomato. The effect of root exudates from plants with and without nematode infection must be examined to see if there are chemical cues that influence the pathogenicity of the fungus against nematodes.

Key Words: *Pochonia chlamydosporia*, tri-trophic interaction, nematophagous

Introduction

The nematophagous fungus *Pochonia chlamydosporia* (formerly *Verticillium chlamydosporium*) has been developed as a biological control agent (BCA) for root-knot and cyst nematodes. The fungus is applied as multi-nucleate chlamydospores from which it colonises the rhizosphere. When the fungus encounters nematodes, it forms appressoria on the egg surface under which it secretes extracellular enzymes, particularly the protease VCP1 (Segers *et al.*, 1994) and chitinases (Tikhonov *et al.*, 2002). Through the combination of enzymolosis and mechanical pressure, the fungus penetrates the eggshell and colonises the developing nematode juvenile. After consuming the nematode the fungus exits the egg and produces greater numbers of chlamydospores than produced during saprophytic growth (Kerry & Crump, 1998).

When infecting the nematode egg mass from the rhizosphere, the fungus can be influenced by both hosts. There has been some indication that there is a critical distance at which the fungus can detect the egg mass (Biram, 2003). There is host-related genetic variation in *P. chlamydosporia* (Morton *et al.*, 2003) and plants differ in their ability to support fungal growth and differ in their ability to act as nematode hosts (Kerry & Bourne, 1996). This indicates that there are specific interactions between the organisms in the tri-trophic interaction.

It is not known if the plant influences the fungus to infect the nematode egg mass or whether the fungus is attracted to the egg mass. The aim of this report is to show that the plant host has an effect on the fungus and to discuss previous findings on the tri-trophic interaction with regard to current research.

Materials and Methods

Fungal cultures
Isolates of *P. chlamydosporia* were obtained from the Rothamsted culture collection and maintained on corn meal agar (Oxoid) in darkness at 28 °C.

Plant Growth and Exudate Collection
Lycopersicon esculentum var. Tiny Tim (Tomato) and *Brassica oleracea* var. Greyhound (Cabbage) seeds were sterilised in 75 % ethanol followed by 7 % (w/v) calcium hypochlorite for 15 min and washed 5 times in distilled water. The seeds were grown in sterile sand in sterile Magenta vessels (Sigma) for 10 days in darkness. The germinated seedlings were transferred to the Life Raft System (Sigma) containing 250 ml B5 medium (KNO_3, 2.5 g l^{-1}; $(NH4)_2SO_4$, 0.134 g l^{-1}; $MgSO_4.7H_2O$, 0.25 g l^{-1}; $CaCl_2.2H_2O$, 0.15 g l^{-1}; $FeSO_4.7H_2O$, 0.0278 g l^{-1}; $MnSO_4.4H_2O$, 0.01 g l^{-1}; $ZnSO_4.7H_2O$, 0.002 g l^{-1}; $CuSO_4.5H_2O$, 0.00003 g l^{-1}; $CoCl_2.6H_2O$, 0.00002 g l^{-1}; KI, 0.00075 g l^{-1}; H_3BO_3, 0.003 g l^{-1}) for 5 weeks. Exudates were collected from the Life Raft containers and filtered using 0.2 µm Nalgene filter units. These were stored at –80 °C until required.

Fungal Growth Experiment
A single plug of fungal mycelium (4mm) was transferred to the centre of a Petri dish containing water agar (1.5 %) amended with 25 % (v/v) root exudates from either tomato or cabbage. The plates were incubated in darkness at 28 °C until the mycelium had reached the edge of the Petri dish. Fungal radial growth was determined by measuring the colony diameter and hyphal density determined by counting the number of hyphae per mm of colony perimeter at X100 magnification.

VCP1 Induction Experiment
Mycelium was grown in 300 ml Czapek Dox broth at 28 °C with shaking in a Gallenkamp orbital shaker at 110 rpm. The mycelium was harvested after 10 days by filtration through muslin. This mycelium was divided and aseptically transferred to 100 ml conical flasks containing SDW. This was incubated overnight under the same conditions. Flasks were amended with one of the following solutions; tomato root exudate, B5 medium or SDW. These were incubated for 3 days as before and a sample of culture medium was take each day. VCP1 activity was measured as described by Segers *et al.* (1994).

Results and Discussion

Pochonia chlamydosporia is affected by root exudates. The data in Fig. 1 indicate that hyphal density is less on media amended with cabbage root exudates. The mycelium grown on cabbage root exudates was very sparse although its radial growth rate was the same as mycelium grown on tomato root exudate. This contrasts to data obtained by Kerry & Bourne (1996) that showed that there was a greater abundance of *P. chlamydosporia* on roots of kale and cabbage compared to those of tomato. This supported a biological control strategy of increasing the amount of *P. chlamydosporia* in soil by planting kale before tomato. However, the data presented in this paper indicate that fungal proliferation should be greatest under tomato. The apparently contradictory results can be explained if one considers that *Brassica* species produce antimicrobial compounds (Brabban & Edwards, 1995) whereas tomatoes do not exhibit the same antimicrobial activity. It was shown in a pot test where *P. chlamydosporia* was applied as a BCA that microbial biomass and diversity was greater under

tomatoes than under cabbage, however, there was more *P. chlamydosporia* recovered from cabbage than tomato (Ian Clark, Pers. Comm.). It must also be considered that the effect of exudates in assays on agar plates at 25 % (v/v) may not reflect of conditions in the rhizosphere. There are also isolate specific differences in rhizosphere competence. The rhizosphere of cabbage may create an environment which *P. chlamydosporia* can tolerate allowing it to grow with relatively few competitors. In the tomato rhizosphere *P. chlamydosporia* was outcompeted by other faster growing microbes and thus could not establish itself as well. This shows that the plant being treated must be considered before applying a BCA since it actively influences the environment in which the BCA must act. Another factor that might influence the ability of plants to support fungal growth is the composition of the exudates. Tomato exudates may simply contain more available nutrients than cabbage root exudates and root architecture may play a role in how exudates are distributed in the rhizosphere. There is a need for further study on the chemical composition and distribution of exudates.

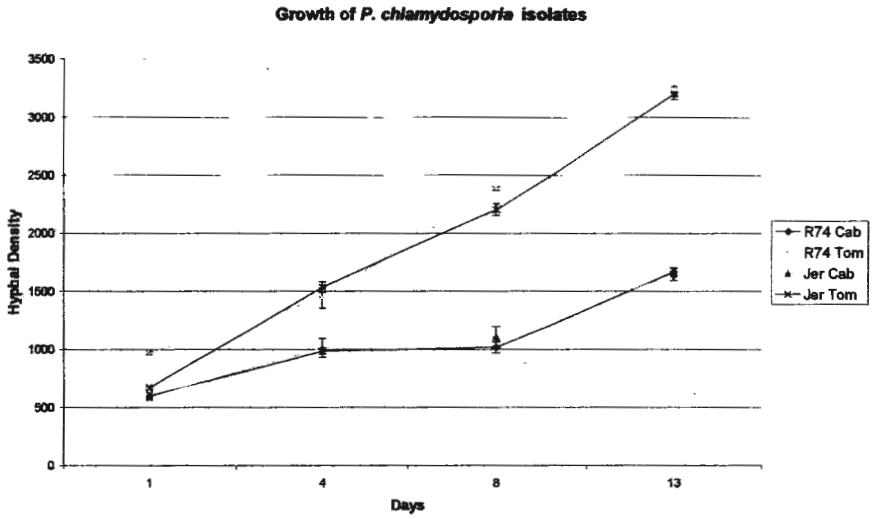

Figure 1. Growth of *P. chlamydosporia* isolates, Res 74 and Jersey, on water agar amended with 25 % tomato (Tom) or cabbage (Cab) root exudates. Both isolates have a greater hyphal density on agar amended with tomato root exudate.

The ability of *P. chlamydosporia* to tolerate a variety of antibiotics and fungicides has been examined (Atkins, 2000) and can now be considered in terms of the ecology of the fungus. The nematode egg mass is thought to be antimicrobial in nature but it still supports a variety of microbes. In this environment, *P. chlamydosporia* must survive microbial antibiotics and the chemicals that constitute the egg mass (Kok *et al.*, 2001). The ability to survive in the cabbage rhizosphere (Brabban & Edwards, 1995) is another example of the competitive advantage of its toxicity tolerance. In inhospitable environments the slow growing tolerant fungus has an advantage but there is an apparent fitness cost, the fungus is not a competitive saprophyte in less toxic environments. Fitness costs have been demonstrated in pesticide resistant *Myzus persicae* where resistant aphids were more prone to parasitism (Foster *et al.*,

2003). The evolution of facultative parasitism in *P. chlamydosporia* may be a response to its inability to compete as a saprophyte. To survive, the fungus must occupy ecological niches where it has competitive advantages. The biological control tri-trophic interaction is an ecological niche which *P. chlamydosporia* can effectively exploit. For successful application of the fungus as a BCA it must be directed towards nematode parasitism. This might explain why there are reports that the presence of root-knot nematodes can increase the abundance of *P. chlamydosporia* on tomato roots. It may be possible to manipulate plants to maintain the fungus in a parasitic state once the factors controlling the switch to parasitism have been elucidated.

Tomato root exudates may have an effect on gene regulation in the fungus (Figure 2). The data are very preliminary but there appears to be an effect of root exudates in increasing VCP1 expression ($P<0.05$) indicating that the plant could influence fungal virulence. This requires more rigorous investigation.

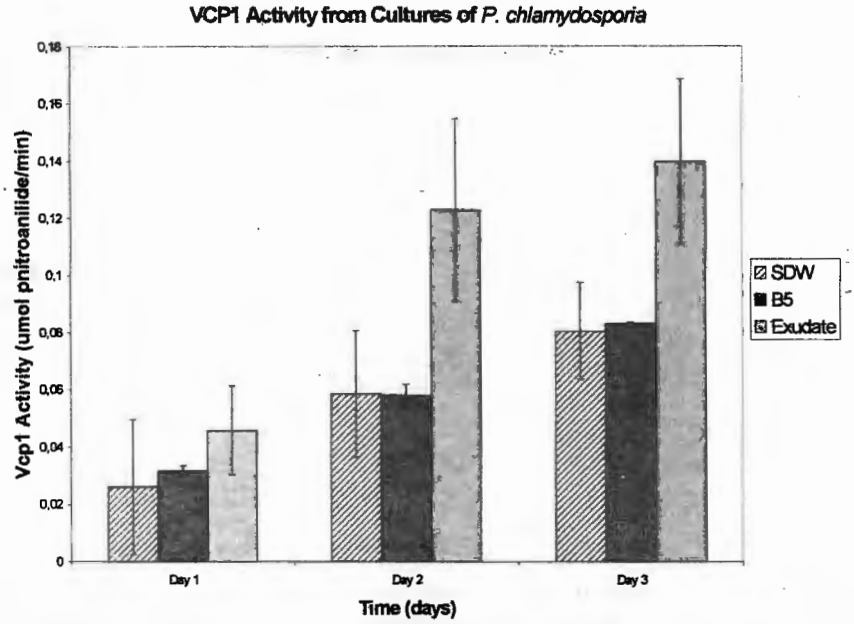

Figure 2. Induction of VCP1 activity in mycelia exposed to tomato root exudates. VCP1 activity was measured using the chromogenic substrate Succ-$(Ala)_2$-Pro-Phe-pNa at 410 nm.

Biological control agents such as *P. chlamydosporia* are not be able to adequately replace pesticides such as methyl bromide and must be used as part of integrated management strategies. A greater understanding of the interactions of BCAs in the systems to which they are applied will allow the development of strategies that will allow maximisation of the potential of BCAs.

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Strategies in developing an efficient commercial product for biological control of soil borne fungal pathogens by *Serratia plymuthica* HRO-C48

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Abstract: The object of this publication is to describe the development of a commercial product for biological control of soil borne fungal pathogens by *Serratia plymuthica* strain HRO-C48. The strain was evaluated for disease reduction and plant growth promotion of several crops (strawberry, oilseed rape, lettuce and sugarbeet) in greenhouse trials. Consecutive field trials in five vegetation periods on strawberries resulted in reduced Verticillium wilt and increased yield compared to the untreated plants. Simultaneously, rhizosphere competence of *Serratia plymuthica* was confirmed by cultivation dependent und cultivation independent approaches. A solid and a liquid formulation for *S. plymuthica* were evaluated by testing cell counts and stability of antifungal mechanisms, plant growth promoting effects as well as genetic stability. No change in of these properties was observed after storage for three month at 4°C. An enhancement of the stability of formulation is aimed for further developments. Addition of osmoprotective substances to the formulation and changing of cultivation conditions are planned. Furthermore the field of application will be extended to other crops,e.g. oilseed rape and olive trees.

Key words: *Serratia plymuthica* C48, *Verticillium*, biological control, strawberry, oilseed rape

Introduction

The fungus *Verticillium dahliae* Kleb is a soil borne cosmopolitan fungal pathogen with a broad host spectrum of over 150 plants. *V. dahliae* causes Verticillum wilt connected with high yield losses in strawberry, olive, oilseed rape and cotton. From the year 2003 the fungus has been classified as high-risk pathogen. In the future, there is no possibility controlling this pathogen due to the lack of chemical control by the ban of methyl bromide.

An enviromentally friendly alternative to protect roots against fungal pathogens is rhizobacteria-mediated biological control. *S. plymuthica* strain HRO-C48 (German Collection of Microorganisms and Cell Cultures: DSMZ 12502) was isolated from the rhizosphere of oilseed rape and selected as a biocontrol agent according to the following criteria: (a) high antifungal activity against fungal pathogens, e.g., *V. dahliae* and *Phytophthora. cactorum* in vitro, (b) production of the plant growth hormone indole-3-acetic acid, (c) relative harmlessness to human health and the enviroment; and (d) low level of antibiotic resistence (Berg 2000).

The plant protecting and plant growth promoting effect on strawberries caused by *S. plymuthica* HRO-C48 have been patented (EU patent 98124694.5.). The E-nema GmbH, company for plant protection and biotechnology (Raisdorf, Germany) will produce and distribute the product, called Rhizostar®. This paper marks the milestones of development of the biofungicide based on *S. plymuthica* and presents an outlook for further developments.

Past developments

Field experiments on strawberries

Five field trials at different locations in cooperation with the strawberry farm in Röverhagen (Germany) were carried out to evaluate the ability of *S. plymuthica* C48 to promote plant growth of strawberries and suppress the pathogens under natural conditions. Dipping plant roots in a suspension of *S. plymuthica* prior planting reduced symptoms of Verticillium wilt compared to the non-treated control by 0 to 38%, to average of 24% (Kurze et al. 2001). The yield increased by 0 to 300%, to average of 44%. The reduction of Phytophthora root rot by *S. plymuthica* treatment with ranged from 2 to 18%.

In general, a high biocontrol activity was correlated with an high yield enhancement. Increased yield was not observed when disease reduction was absent. The varying results were strongly dependend on the initial pathogen inoculum, but also on abiotic factors, e. g. weather conditions and soil parameters.

Rhizosphere competence

An efficient biological is required to be able to establish in the rhizosphere (Lugtenberg et al. 2001). To demonstrate the rhizosphere competence of *S. plymuthica* two approaches were carried out. The reisolation and cultivation of rifampicin-resistant mutants from the rhizosphere at levels of approximatly 3 to 7 $\log_{10}$ CFU g^{-1} over the period of of 14 month under field conditions showed the ability of *S. plymuthica* to colonize the roots of strawberry plants (Kurze et al. 2001). As a cultivation independent method to determine the rhizosphere competence and the influence on the autochthoneous bacterial communities the Single Strand Conformation Polymorphism analysis (SSCP) based on 16S rRNA genes was used (Schwieger & Tebbe 1998). Compared to SSCP patterns of the non-treated samples an additional band appeared in the patterns of treated samples which corresponded to *S. plymuthica* HRO-C48. Till now a shift in the bacterial community due to the treatment was not detected.

Formulation

Serratia plymuthica HRO-C48 was formulated in (a) a solid matrix consisting of silica powder, and (b) in a liquid matrix consisting of a vegetable oil and an emulsifier. After storage for three month at 4°C and 20°C both formulations were evaluated reagarding the viability of bacteria and stability of the antifungal activity, plant growth promoting effects and genetic properties. During storage at 4°C in both formulations the cell count were stable at a level of 9 $\log_{10}$ $CFUg^{-1}$ and $CFUml^{-1}$ respectively. No loss or changes of the mentioned attributes were observed. After storage at 20°C no viable bacteria were detected in the solid formulation. From the liquid matrix only 1% of the initial cell count could be recultivated.

Further developments

Results from the shelf-life tests suggest a deficient stability of formulations at 20°C. Our aim is to extend the shelf life to at least six month at room temperature. Therefore the influence of several protective compounds on the survival of *Serratia plymuthica* will be tested. As protection against dehydration osmoprotective substance e.g. trehalose, sucrose and glucosylglycerol could serve. Cultivation conditions can affect the stress tolerance of bacteria (Wessendorf & Lingens 1989). Experiments on pre-adaption to suboptimal conditions, e.g. via cultivation at low water-activities, will be carried out.

At present, Rhizostar® is applied to control pathogenic fungi on strawberry. Soil borne fungi, especially *Verticillium dahliae* also causes dramatic damage in oilseed rape and olive trees, so an application of *S. plymuthica* to these crops is reasonable. Results from greenhouse experiments with oilseed rape in *V. dahliae* inoculated soil showed statistically significant disease reduction and plant growth promotion after seed treatment with *S. plymuthica* (Frankowski et al., 1998). The evaluation of the pathosystem olives - *V. dahliae* is planned for future experiments. Different from treating strawberry plants, the bacterial inoculum has to be applied to root of oilseed rape and cotton by using adapted technologies. For oilseed rape a seed-coating will be developed which can be reproduced on high throughput machines of the seed companies. In olive farms constructions for drip-irrigation are used. The possibility using existing facilities to treat the roots of olives with *S. plymuthica* will be proved.

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Can fungal endophytes control soilborne pests in banana?

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Abstract: A variety of endophytic fungi were isolated from banana corm and root tissue and screened in vivo for activity against banana weevils, nematodes (*Radopholus similis*) and Fusarium wilt, key pests and diseases of banana. The most promising strains were selected for inoculation onto tissue culture banana plants. The selected strains were able to colonize tissue-culture banana when plants were inoculated at an early stage. Inoculation of endophytes could lead to, depending on fungal isolate and banana clone, smaller weevil larvae, reduced weevil damage and reduced nematode multiplication. However, inoculation of endophytes did not reduce disease expression of Fusarium wilt in these experiments. The results provide evidence on the potential for use of fungal endophytes for banana weevil and nematode management during early plant developmental stages of banana. Long-term pest and disease management might involve persistence of the fungal endophyte or the activation of effective and durable plant defense responses in bananas. Use of *Fusarium oxysporum* endophytes for the management of Fusarium wilt of banana appears to hold less promise.

Key words: Banana, tissue culture, endophytes, *Fusarium oxysporum*, banana nematode, *Radopholus similis*, banana weevil, *Cosmopolites sordidus*, Panama disease

Introduction

The key pests of banana (*Musa* spp.) in the East African highlands are the banana weevil, *Cosmopolites sordidus* (Germar) and plant parasitic nematodes, particularly the migratory endoparasite *Radopholus similis* (Cobb) Thorne (burrowing nematode). The major soilborne disease of banana is Fusarium wilt caused by *Fusarium oxysporum* Schlecht. f. sp. *cubense* (E.F. Smith) Snyd. and Hans. These pests and disease attack the roots, corm and vascular system of the plant, weakening the stability of the plant and interfering with water and nutrient uptake. Damaging stages of the banana weevil and root nematodes occur inside banana root and corm tissue and dissemination is primarily through the movement of infested planting material (Gold et al., 2002; Gowen and Quénéhervé, 1990). Clean planting material, including tissue-culture plantlets, has been widely promoted as a means of reducing the spread of banana weevil, nematodes and Fusarium wilt (Speijer et al., 1999; Gold et al. 2002). Pest and disease reinfestation, however, remains a critical concern. Gold et al. (1998) found the use of paring and hot-water treatment to reduce but not eliminate banana weevils in treated suckers. Hot water treatment furthermore does not eliminate the banana wilt pathogen.

The International Institute of Tropical Agriculture (IITA), in collaboration with the University of Bonn, Germany, has been conducting research on control methods targeting the damaging stages using fungal endophytes and to extend the life of clean planting material in banana. Fungal endophytes are organisms that live within plant tissues without causing symptoms of disease (c.f. Petrini, 1991). Endophytes can be isolated from roots, stems, corms and leaves and the presence of endophytes has been demonstrated in all plants investigated including important crops such as banana, maize, rice, and tomato (Fisher et al., 1992; Fisher and Petrini, 1992; Hallmann and Sikora, 1994; Brown et al., 1998; Pereira et al., 1999). The

relationship between plant and endophyte can be mutualistic and some endophytes act as antagonists to pests and diseases. The best documented examples of biological protection using fungal endophytes are those of the grass-endophytes (Clay, 1990, 1991). More recently, the potential of endophytes to control banana weevil and nematodes was demonstrated by Pocasangre (2000), Griesbach (2000), Niere (2001) and Sikora et al. (2002).

The objectives of this study were to test the effect of fungal endophyte inoculation on damage and multiplication of pest organisms of banana and to evaluate their potential as a pest management option for tissue-cultured banana plants.

Material and methods

Plant material

Banana cultivars used in experiments for nematode and banana weevil control were Enyeru (*Musa* AAA-EA, Nfuuka clone set) and Kibuzi (*Musa* AAA-EA, Nakabululu clone set), respectively. The cultivar Kayinja (*Musa* ABB, Pisang Awak group) was used in experiments for Fusarium wilt control. Plants used in all experiments were micropropagated in tissue culture. The protocol for micropropagation followed the method for banana shoot tip culture described by Vuylsteke (1998). Cultures were initiated from explant material collected from the IITA germplasm collection at Namulonge, Uganda. After initiation of an aseptic culture, subculturing for multiplication was undertaken every 4 weeks. At the last stage, shoots were transferred to rooting medium for root initiation. Plants were singly rooted in test tubes and kept in laboratory incubators at 27 °C under a light/dark-cycle of 14/10 h. Postflask management followed the recommendations of Vuylsteke and Talengera (1998) but modified for endophyte inoculation.

Fungal isolates and endophyte inoculation

The fungal endophytes used were isolated from healthy banana root and corm tissue and identified as Fusarium oxysporum (Schuster et al., 1995; Griesbach, 2000; Niere; 2001). None of the isolates caused disease on banana or was vegetatively compatible with all known tester strains for F. oxysporum f. sp. cubense (Niere, 2001). Isolates were selected on their basis to immobilize or cause mortality of nematodes or weevil eggs and larvae under in vitro conditions, and their non-pathogenicity to banana (Schuster et al., 1995; Griesbach, 2000; Niere; 2001).

Cultures were preserved on soil tubes and revived on synthetic nutrient-poor agar (SNA). Fungal spores for inoculation onto tissue-cultured banana were multiplied in Erlenmeyer flasks containing 200 ml of Potato Dextrose Broth (PDB). Flasks were incubated at 22-26 °C under natural light conditions and shaken manually once per day. Spores were harvested by filtering the fungal slurry through a KLEENEX tissue placed in a filter holder. Spore densities were estimated using a hemacytometer (Thoma-Kammer) and suspensions of $1.5*10^6$ spores/ml were used for inoculating tissue cultured banana plantlets.

Plants singly grown in test tubes were selected for homogeneity of size and removed from the tubes. The roots were washed free of medium and cut back to approx. 2 cm in length. Plants were then dipped in the spore suspension or PDB as control treatment. After five to 15 min during which the plantlets were occasionally shaken in the suspension, the plants were potted in plastic pots with drainage holes containing 150 ml of steam-sterilized soil. The pots were maintained in a humidity chamber on trays separating treatments and were watered and misted regularly. After a 4-week hardening period, the plants were transferred to the greenhouse and transplanted into polythene bags containing 3 l of steam sterilized soil. Plants used in experiments for weevil control were transferred to 20 l buckets after 4 months while

plants used in Fusarium wilt control experiments were transferred to 10 l buckets with infested soil.

Challenge inoculation and infestation

Adult banana weevils (*Cosmopolites sordidus*) were collected in banana fields and reared on banana corm pieces in covered plastic containers in the laboratory. Five adults were released near the pseudostem base of 8 month-old banana plants grown in 20 l buckets filled with steam sterilized soil (modified from Griesbach, 2000). The plants were then covered with a mosquito net to confine weevils in the buckets by fastening the nets to the buckets and the pseudostems with rubber bands; drainage holes in the buckets were also covered with netting material.

Pure nematode cultures of *Radopholus similis* were multiplied on surface sterilized carrot disks in 40 mm glass Petri dishes ((Speijer and De Waele, 1997). Nematodes from the carrot disk cultures were extracted with tap water and 500-1,000 *R. similis* (mixed stages) were inoculated onto 4 month old banana plants previously transferred to polythene bags.

Fusarium oxysporum f. sp. *cubense* isolate FOC 4-C was isolated in Uganda from the corm of infected banana plant (cv. Kivuvu, *Musa* ABB, Bluggoe subgroup) which proved highly virulent in greenhouse experiments on Gros Michel, *Musa* AAA (Niere et al., unpublished). The fungal isolate for inoculation was multiplied in 10 l plastic buckets filled with an autoclaved soil-maize bran mixture (9:1 v/v). Spore suspensions for inoculation of the soil-maize bran mixtures were produced in PDB and mixed thoroughly into the soil. The buckets were covered and the fungus incubated for 10 days before planting bananas in the infested soils.

Data collection and analysis

Plant growth parameters (pseudostem height, number of leaves, and leaf area of the youngest leaf) were measured on a monthly basis over the trial duration. Additionally, number of dead and functional roots, root fresh weight and aerial plant parts, damage and multiplication/development of pest organisms were recorded at the termination of the experiments.

Weevil damage and larval development was assessed after 4 and 6 weeks after weevil infestation, respectively. The netting material was removed and plants uprooted. The number of adult weevils, eggs and larvae present in soil and plants were recorded from the surface of the pseudostem base and within the corm after sectioning of the corm. Cross sections were made at the base of the pseudostem, and 5 and 10 mm below the pseudostem base section. The percentage of tissue damaged by weevil larvae for both the central and outer cortex of the corm was scored as described by Gold et al. (1994). Extracted larvae were weighed and individually placed in Eppendorf tubes with a few drops of water. Head-capsule measurements were recorded using a counting slide with a drop of water under a microscope fitted with an eyepiece micrometer.

Nematode damage and multiplication was assessed at 8 weeks after nematode inoculation. Plants were uprooted and ten roots per plant were chosen at random, cut to 10 cm and split longitudinally to assess the percentage of necrotic root tissue. Each split root segment of 10 cm was estimated to be 10 % of total root tissue assessed. The necrotic tissue of the ten segments was then calculated according to Speijer and Gold (1996). The roots used for damage assessment were chopped into small pieces of approx. 4 mm length, a sub-sample of 5 g removed and macerated in a Warring Blender for 2 x 10-15 s at high speed and nematodes extracted overnight using a modified Baerman funnel technique described by Hooper (1990). Nematode densities were assessed from the nematode suspension, adjusted to

25 ml. Aliquots of 2 ml were counted three times in a counting slide. Number of females, juveniles, and males were recorded separately.

Vascular discoloration caused by *Fusarium oxysporum* f. sp. *cubense* was evaluated following the INIBAP guidelines No. 3 (Orjeda, 1998). Due to the small size of the rhizome, 3 instead of 5 cross sections were made. Discoloration was ranked on a scale of 1 (no discoloration) to 6 (total discoloration of vascular tissue) according to (Orjeda, 1998).

Results and discussion

Banana weevil larvae recovered from banana plants inoculated with fungal endophytes were up to 25% smaller than larvae from control plants as measured by head capsule size (Figure 1) in our experiments. This effect was observed after 4 and 6 weeks challenge with 5 banana weevil females (Figure 1). Larvae were considerably larger after 6 weeks compared to 4 weeks challenge period, however, percentage reduction in size of larvae was similar after 4 and 6 weeks. More important than the effect of endophyte inoculation on weevil larvae size was the reduction in damage indices. Endophyte inoculation onto tissue cultured banana resulted in up to 70% less damage in inner corm tissue and 40-50% reduction in damage indices in outer corm tissue (data not shown). This is an important finding which clearly demonstrates the suppressive effect of endophyte inoculation on development and activity of banana weevil larvae in vivo.

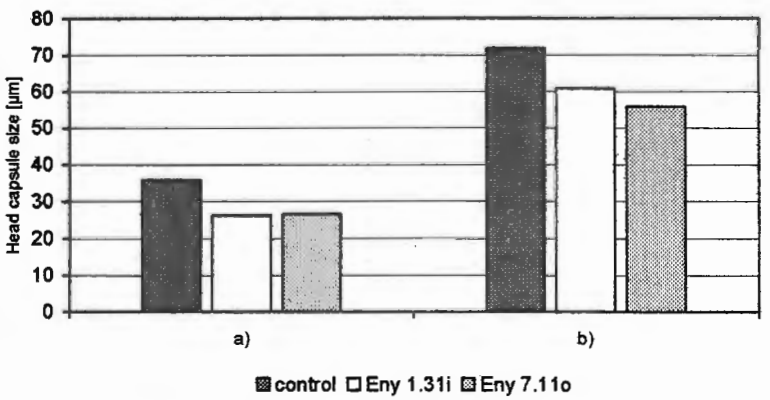

Figure 1: Head capsule size of banana weevil larvae recovered from 38-week old *Musa* AAA EA cultivar Kibuzi plants inoculated with fungal endophytes 4 (a) and 6 weeks (b) after infestation of banana plants with 5 *Cosmopolites sordidus* females. Adapted from Kapindu et al. (unpublished data)

Fungal endophyte inoculation onto tissue cultured banana prior to infestation with *R. similis* could result in reduced nematode multiplication in the roots, reduced root damage expressed as percentage root necrosis, and in some instances lead to increased plant growth parameters (Athman et al., unpublished). On the banana cultivar Nabusa (*Musa* AAA-EA), nematode multiplication was suppressed by 30-40% over the control following inoculation with the fungal endophytic isolate V5w2 (table 1). Although effects on multiplication, damage and plant growth (data not shown) following fungal endophyte inoculation could be observed,

differences were not in all cases significantly different (Athman et al., unpublished). In none of the experiments were nematode numbers in endophyte treated plants higher than in the control plants.

Table 1: Numbers of *Radopholus similis* per gram of root of 16-week old *Musa* AAA-EA cultivar Nabusa inoculated with *Fusarium oxysporum* isolate V5w2 8 weeks after *Radopholus similis* challenge inoculation

Treatment	Mean number of *Radopholus similis* per g root				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Control	17	388	163	77	334
V5w2	11	378	108	52	197
Change	-35%	-3%	-34%	-32%	-41%

Adapted from Athman et al. (unpublished data)

A trend of reduced multiplication and root damage caused by *R. similis* was observed in most experiments (experiments 1, 4, and 5) compared to control plants (table 2). However, percentage necrotic root tissue in experiments 2 and 3 was not affected. Although, the mechanisms leading to lower nematode multiplication are unclear, these results clearly demonstrate that fungal endophyte inoculation can result in a reduction of *R. similis* in the roots of banana.

Table 2: Nematode damage caused by *Radopholus similis* expressed as percentage necrotic root tissue in roots of 16-week old *Musa* AAA-EA cultivar Nabusa inoculated with *Fusarium oxysporum* isolate V5w2 8 weeks after *Radopholus similis* challenge inoculation

Treatment	Root necrosis caused by *Radopholus similis*				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Control	18.2	12.4	24.1	18.6	32.3
V5w2	11.7	11.9	26.3	15.3	25.3
Change	-36%	-4%	9%	-18%	-22%

Adapted from Athman et al. (unpublished data)

In the current study, inoculation of non-pathogenic strains of *F. oxysporum* did not affect Fusarium wilt. Although plant growth was higher in some endophyte inoculated plants compared to plants challenged with the pathogen only, internal discoloration was only slightly reduced and could not contribute to plant health improvement (data not shown). Similar to control plants, internal discoloration ranged between 5 and 6 (total discoloration) when plants were challenged with the highly aggressive strain of *F. oxysporum* f. sp. *cubense* FOC 4-C. Whether the lack of Fusarium wilt control in our experiments is attributed to the aggressiveness of the pathogenic strain or to an ineffectiveness of the endophytic fungi used here requires further assessment.

However, it was clearly demonstrated that fungal endophytes may offer biological protection against banana weevil and nematodes for at least initial plant growth stages as supported by other studies (Pocasangre, 2000; Griesbach, 2000; Niere, 2000; Kapindu et al., unpublished; Athman et al., unpublished). Although the mechanisms of control are not yet understood, this protection could increase plant health of banana. Whether this is sufficient to give long-term control effects in the field is currently investigated at IITA in on-station experiments.

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Soil Organic Matter

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Introduction

When we think about soils we generally accept that most soils have an organically enriched topsoil, although we recognise that the amount of this enrichment may vary considerably

When we characterise soil we normally do so under four major headings; Mineral Matter; Organic Matter; Air; and Water. The proportions of these vary, but on a volumetric basis it is likely that in a cultivated topsoil the proportions would have been of the order:

45% Mineral Matter;
5% Organic Matter and
50% pore space

In a natural soil this organic matter will consist of living plant and animal material, dead plant and animal material, where the origin of the material is clearly discernible, and well decomposed organic material called Soil Organic Matter (SOM).

This SOM fraction will consist of: -

1. partially decayed plant residue (no longer recognisable as plant material),
2. micro-organisms and microflora involved in decomposition
3. by-products of microbial growth and decomposition
4. humus where the by-products have undergone humification

The humus material will often be expected to consist of

50-55% Carbon,
4-5% Nitrogen and
1% Sulphur

It is a relatively stable component of the organic fraction which may persist for a number of years, particularly when in intimate association with components of the mineral fraction. When in intimate association with fine silt and clay fractions the organic material will persist for a number of years.

Organic matter additions to soils have long been considered important in maintaining the 'quality' of soils because of their role in providing nutrients and through their effect on physical properties.

Before the widespread advent of manufactured fertilisers organic residues were the only means of adding substantial proportions of many plant nutrients to the soil.

This was particularly the case with respect to Nitrogen. In non-cultivated soils it is likely that more than 95% of the Nitrogen and Sulphur is found in the soil organic matter, and possibly as much as 25% of the Phosphorus.

Organic materials together with fine clays and amorphous and crystalline inorganic materials provide the cementation between the coarser fragments to stabilise the aggregates and the pore space of the soil, organic materials also have pore characteristics that provide the capability to absorb water, and their persistence (recalcitrance or protection against attack by microorganisms) is important (Kay, 1998)

Whilst with the introduction of manufactured fertilisers there has been a reduction in the reliance upon organic residues as the principal source of nutrients, with substantial additions of nutrients to the soil as inorganic fertilisers. There are however major differences in the nature of nutrient provision from organic and inorganic fertilisers.

In particular organic sources of nutrients are generally relatively slowly released for plant uptake. The rate of nutrient release is controlled by: -

1. The nature of the materials; and
2. The conditions prevailing in the soil.

For example low ratios of Carbon to Nitrogen and Carbon to Phosphorus in the residues tends to result in faster rates of release of nutrients.

What is the target Soil Organic Matter Level?

There has been considerable concern expressed about the decline in the quality of cultivated agricultural soils possibly as a result of modern agricultural practices. Frequently this decline has been associated with a reduction in the levels of organic matter in soils. In the United Kingdom there was a report commissioned in 1970, known as the **Strutt Report** to evaluate the possibility of a decline in soil quality following a series of poorer harvests recorded during the 1960's. This report entitled '**Modern Farming and the Soil**' drew attention to the apparent increased vulnerability to structural damage of many agricultural soils where levels of soil organic matter were declining. This vulnerability was particularly marked where there were adverse (wet) conditions prevailing at key periods in the cropping cycle; land preparation and harvest. In 1996 the Nineteenth Report of the Royal Commission on Environmental Pollution, entitled '**Sustainable Use of Soil**', similarly drew attention to maintain and increase levels of soil organic matter, particularly in agricultural soils. A number of workers have stressed that the maintenance and improvement of soil organic matter content is generally accepted as being an important objective of any sustainable agricultural system, particularly with respect to soil structural properties. There have been suggestions that there might be a critical level of soil organic carbon of approximately 2%.

Further examination between soil behaviour and soil organic matter content suggests that this relationship is complex and variable, particularly with respect to texture and cultivation history.

It has been suggested that soils with high clay content may require a significantly lower level of organic carbon before physical soil quality deteriorates significantly.

A target of 2 to 4% organic carbon in soils appears to be a widely accepted target, although account must also be taken of the vertical distribution of the organic carbon and the depth of the organically enriched layer in any evaluations.

Whilst the level of organic carbon may be a useful broad level indicator, it has been suggested that of far greater importance in determining the actual influence of the organic fraction is the relatively magnitude of the active fraction, or that which is currently undergoing alteration. There is only limited scientific evidence to support this, but if there is a clear relationship between the presence and magnitude of the active fraction of the organic matter, then the aim should not be to target a critical level of total organic matter in the soil,

but rather maintain a base level of soil organic matter, but make regular additions of readily decomposable organic matter to maintain the active pool.

Why is Soil Organic Matter important in Soils?

It has been the claim of many, particularly in the field of organic farming that soil organic matter has key roles in maintaining soil quality and soil health. Without adequate levels of soil organic matter the soil will not be capable of functioning optimally.

For example Haynes (1999) whilst mentioning the importance of soil organic matter as a reservoir of nutrients such as N, S and P, specifically cites the importance of the labile fraction of the organic matter in influencing the aggregate stability of soils.

Table 1. Role of Organic Matter in Soil

Property	Remarks	Effects on Soil
Colour	The typical dark colour of many soils is often caused by organic matter	May facilitate warming in spring
Water Retention	Organic Matter can hold up to 20 times its weight in water	Helps prevent drying and shrinking. May significantly improve the moisture retaining properties of sandy soils. The total quantity of water may increase but not necessarily the AWC except in sandy soils
Combination with clay minerals	Cements soil particles into structural units called aggregates	Permits the exchange of gases. Stabilises structure. Increases permeability
Reduction in the Bulk Density of Mineral Soils	Organic materials normally have a low density, hence the addition of these materials 'dilutes' the mineral soil	The lower bulk density is normally associated with an increase in porosity because of the interactions between organic and inorganic fractions.
Chelation	Forms stable complexes with Cu^{2+}, Mn^{2+}, Zn^{2+} and other polyvalent cations	May enhance the availability of micronutrients to higher plants
Solubility in water	Insolubility of organic matter because of its association with clays. Also salts of divalent and trivalent cations with organic matter are insoluble. Isolated organic matter is partly soluble in water	Little organic matter is lost through leaching
Buffer action	Organic matter exhibits buffering in slightly acid, neutral and alkaline ranges	Helps to maintain uniform reaction in the soil.
Cation exchange	Total acidities of isolated fractions of organic matter range from 300 to 1400 $cmol_c\ kg^{-1}$	May increase the CEC of the soil. From 20 to 70% of the CEC of many soils is associated with organic matter.
Mineralisation	Decomposition of organic matter yields CO_2, NH_4^-, NO_3^-, PO_3^{4-} and SO_2^{4-}	A source of nutrients for plant growth

Soil organic matter also provides a source of energy for soil organisms. Many of the structural qualities of the soil and the release of nutrients are closely associated with the biological activity of the soil. Regular applications of organic material provide a source of energy and nutrients, maintaining the population of soil organisms.
This provision of nutrients from organic sources has been long recognised as a potentially important role for organic materials in the soil, but in addition there are many other key functions which are directly or indirectly related to the presence of soil organic matter, including

Rawls (1983) established a statistical relationship between bulk density and soil organic carbon content and texture based on 2721 soils in the United States of America. The relationship suggests that organic carbon clearly influences bulk density, but the nature of the relationship varies with bulk density.

What are the fractions of the organic residues?

As mentioned above the organic fractions of the soil is diverse ranging from fresh clearly discernible plant material through to humus where there are no visible signs of plants or animals.

When organic matter is added to soil it is subject to decomposition, although rates of decomposition will be influenced by both the nature of the material and the soil environmental conditions.

The process of decomposition and the by-products produced are perhaps the determinants of many of the key roles of the organic materials in soil. The rate of turnover of organic materials varies considerably;

Table 2 presents information for four key fractions of the soil organic matter.

Table 2. Turnover Time of Organic Fractions in Soil

Fraction	Turnover Time (years)
Litter/crop residues	0.5 to 2
Microbial Biomass	0.1 to 0.4
Macro-organisms	1 to 8
Light Fraction	1 to 15

It is however, often difficult to separate the organic residues undergoing decomposition from the soil biota carrying out the decomposition, and the humic substances resulting from the processes.

Organic carbon that is free (has limited association with the mineral fraction) is the lightest fraction. The light fraction may be separated into free particulate organic carbon and occluded organic carbon (requires ultrasonic dispersion of the aggregates to allow separation).

This light fraction is frequently found to be approximately 40% of the organic carbon in cultivated soils. With coarser textured soils the light fraction tends to be a larger proportion of the total organic carbon than in fine textured soils. The light fraction is the fraction that declines most rapidly if limited recycling of organic residues is practices. The light fraction has a key role in stabilising the aggregates.

The heavier fraction of organic materials consists of decomposing plant residues, microorganisms, and extracellular materials. There is a heavier fraction where organic

material is intimately associated with the mineral fraction, which is thought to range from 25% to 50% of the total organic carbon in cultivated soils.

Sources and Composition of Organic Matter additions to Soil

In natural systems the addition of organic matter will consist of plant and animal material as a result of death and exudates through the activity of roots and soil organisms. In agricultural systems there will be the same inputs as in natural systems, but generally at lower rates, and in addition there may be incorporation of crop residues at harvest.

In the past in mixed arable and livestock systems there were additions of farmyard manure, where the rotation involved grass or grass/clover leys there would be a substantial input of organic material at the change from ley to arable farming. In many farming systems involving animals the farmyard manure has often been replaced by a slurry of faeces, urine, and washings from the sheds, potentially a very different material from the manures of the past, with very little carbon.

In recent years there have been introduced a number of other organic materials which have been added to the soil. The most widely used are products of the sewage industry such as sewage sludge, dewatered sewage cake and composted sewage sludge.

Because of concerns raised by the additions of these materials, principally in relation to the possible loading of heavy metals, the additions of sewage sludge to agricultural land have been regulated by national and Europe-wide regulations and directives.

In the past few years the possibility of added other organic residues to soils has been increasingly considered, particularly in the form of composted residues. These materials are wide ranging in origin and in composition, but include:

Food Processing waste Compost;
Green waste Compost;
Municipal Solid Waste Compost (MSW);
Paper waste Compost; and
Industrial waste Compost.

All these materials have Carbon as a major constituent and Nitrogen as a variable constituent (from relatively high levels of N in some food and green waste composts through to very low levels of N in some paper waste composts). The materials would therefore seem to offer the possibility as potential substitutes for normal plant and animal residue additions, there are potential problems.

A major concern with many of these composted materials, as with sewage sludge and sludge-derived composts, has been the heavy metal concentrations in the materials. The Carbon-Nitrogen ratios of a range of materials are presented in Table 3.

Table 3. Carbon-Nitrogen ratios of selected organic materials

Material	C:N
Biosolids (raw)	10
Cow Manure	25
Pig Manure	15
Digested Sewage	6-8
Grass Clippings	15-18
Food waste	18-20
Sawdust	300-500
Paper	500-5000

Incorporating wastes with a high C:N ratio will immobilise Nitrogen from the soil. As a rule of thumb it is possible to suggest that where the nitrogen content is more than 1% there will be mineralisation and release of added N; where the N content is less than 1% it is likely that soil nitrogen will be immobilised.

In addition to the role of the nitrogen content in the compost, in many composts the electrical conductivity (salinity) is high as a result of mineralisation of organic substrates. This salinity is normally readily leachable, but care may need to be taken, particularly if seeds or seedlings are to be planted into the soil compost mix.

On the positive side the composted materials may be a source of nutrients. The ranges of nutrient contributions likely from a wide range of composts are given in Table 4.

Table 4. Approximate concentrations of nutrients in composts

Nutrient	% Dry weight
N	1.0 to 3.0 (except Paper and Wood Waste where levels are much lower)
P	0.4 to 1.0
K	0.5 to 1.5 (water soluble and hence readily lost)
Ca	1.0 to 2.0
Mg	0.5 to 1.0
S	0.5 to 1.0 (lower in paper and MSW)

Once added to the soil the material has to be incorporated into the soil to have beneficial effects. The decomposition of the added compost materials will be dependent upon: -

1. Carbon content and C:N ratio - low Carbon content more rapid decomposition
2. Soil temperature
3. Soil moisture
4. Method of incorporation, generally decomposition is more rapid with materials incorporated into the soil rather than surface applied.
5. The rate of application

What is the strategy for organic matter additions if we have a soil low in organic matter as our starting material?

Under natural conditions the soil will contain organic matter in a wide range of forms from living organic matter, through freshly applied materials, well decomposed materials, to soil humus. The diverse nature of these materials is probably very important in determining the effectiveness of the organic matter in performing many of the roles outlined in Table 1. If however the soil has a low level of soil organic matter and there is a need to increase the level of soil organic matter as part of a planned programme of soil improvement, then there are potentially a number of key considerations to be aware of.

1. A key initial approach will be the rapid release of plant nutrients to stimulated initial plant growth, both to immobilise the readily released nutrients and to provide some protection and stability to the soil surface.
2. In addition to the rapidly released nutrients provide a pool of slow release nutrients to maintain the growth.
3. Provide a substrate for microbial activity, both for the release of nutrients and for development of the organic/inorganic relationships so important in the development of soil physical properties.

Conclusions

Soil organic matter is a key constituent of the soil. The decline in soil organic matter can have a 'knock on' effect with commensurate declines in other soil properties. For good, sustainable soil management it is essential that soil organic matter levels are maintained, and where they have declined markedly a key soil management strategy to maintain the soil system is to undertake soil management policies to increase and maintain the soil organic matter at an optimum level.

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Monitoring gfp-tagged bacterial antagonists in the rhizosphere of tomato plants

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Abstract: Gfp tagging of four bacterial antagonists of *Ralstonia solanacearum* (biovar 2, race 3) was achieved with the IncQ plasmid pSM1890 (Gm, Sm, gfp) which was acquired in triparental matings. The green fluorescent protein was formed in all antagonists (*Pseudomonas putida*, *Enterobacter* sp.) although the colonies showed different brightness of green fluorescence. The presence of the IncQ plasmid was confirmed by PCR with IncQ specific primers. The survival and colonisation patterns of the antagonistic strains in the rhizosphere of root inoculated tomato plants were monitored by selective plating and confocal laser scanning microscopy. Selective plating indicated that all strains had a good rhizosphere competence and were still detectable 29 days after inoculation at levels between 10^6 and 10^7 cells per gram of root fresh weight. In contrast, confocal laser scanning microscopy (CLSM) analysis revealed that only rather few green fluorescent cells were detectable at this time point which might indicate a low metabolic activity of the inoculant strains. Five days after inoculation the roots showed large numbers of green cells and similar colonisation patterns were observed for all antagonists.

Key words: gfp, *Ralstonia solanacearum* antagonists, tomato rhizosphere, colonisation, CLSM

Introduction

In the future, biological control of soil-borne fungal or bacterial pathogens will be of increasing importance for a more sustainable agriculture. This has prompted the search for reliable antagonists which show a high degree of competitiveness and are active in the rhizosphere of different crops and in different soil types. Numerous studies have demonstrated the ability of several rhizobacteria to suppress diseases caused by fungal or bacterial plant pathogens. However, to fully exploit the potentials of biological control agents (BCA) a better understanding of the molecular mechanisms of antagonism, their colonisation patterns and activity in the rhizosphere is required. Mechanisms of bacterial antagonism towards plant pathogens include the competition for nutrients and space, the production of antibiotics, or the production of fungal cell wall-degrading enzymes or induced resistance (Bloemberg and Lugtenberg, 2001). One of the difficulties in developing rhizobacteria as a viable alternative is that many biological control agents are found to be too variable in their performance. According to Raaijmakers and Weller (2001) variable expression of genes involved in disease suppression and poor root colonisation are the major factors contributing to this inconsistency. Successful biological control requires not only a better understanding of the complex regulation of the metabolite production by antagonists in response to environmental factors but also a better picture of what triggers root colonisation and the dynamics and composition of bacterial rhizosphere communities. The dynamics of bacterial

and fungal communities in the rhizosphere of maize grown in tropical soils was recently studied by T/DGGE analysis of 16S or 18S rDNA fragments amplified from DNA which was directly extracted from bulk or rhizosphere soil (Gomes et al., 2001, 2003). The study showed that the relative abundance of bacterial and fungal populations in the rhizosphere changes during plant growth development. During one growth period of maize 50 randomly selected colonies grown on King's B agar per sampling time were isolated. A total of 150 isolates was characterised by their physiological properties (API 20 NE), fatty acid methylester and 16S rDNA sequencing and BOX patterns (Costa et al., in preparation). Antagonistic properties towards different phytopathogenic fungi (*Phytophthora cactorum, Sclerotinia, Rhizoctonia solani*) and *Ralstonia solanacearum* (biovar 2, race 3), antibiotic production and generation of extracellular enzymes (chitinases, proteases) were determined (Costa et al., in preparation).

Here we report a first study on the ability of five selected rhizobacteria which showed strong *in vitro* antagonism against *Ralstonia solanacearum* (biovar 2, pathovar 3) to colonise tomato plant roots. For monitoring the inoculants selective cultivation and *in situ* detection by CLSM were used. The aim of the work was to set up the tools which allow a reliable monitoring of the colonisation and activities of inoculant strains.

Materials and Methods

Strains

The rhizobacterial strains which were tested for their colonisation ability are compiled in Table 1. Furthermore, Table 1 provides data on the identification and antagonistic properties of the strains which were determined as previously described by Berg et al. (2002).

Table 1. Characteristics of *R. solanacearum* antagonists isolated from the rhizosphere of maize

Strains	*R. solanacearum* B3B	*R. solanacearum* 1609	Siderophore	Protease	antbiotic	Chitinase	FAME
PRF 116	12 mm	10 mm	+	-	+	-	*Escherichia vulneris** (55.8%)
PRD 16	6-7 mm	10 mm	+	++	+	-	*Pseudomonas. putida* (58.0%)
PRD 18	4 mm	6 mm		-	+	-	*Pseudomonas putida* (65.2%)
PRD 38	10 mm	4-6 mm	++	++	-	-	*Pseudomonas putida* (87.3%)

*Sequencing of the first approx. 700 bp (single strand) revealed that PRF116 had the highest similarity with endophytes from maize, *Pantoea agglomerans* and *Enterobacter cloacae* (97.7%).

Generation of rifampicin resistant mutants and gfp labelling

Overnight cultures of all four strains were plated on PCA-supplemented rifampicin (50 µg/ml) to select spontaneous rifampicin resistant mutants. The rifampicin resistant mutants were tested for *in vitro* antagonism against *R. solanacearum* B3B and 1609 as described by van Overbeek et al. (2001). The rifampicin resistant mutants were used as recipients in a triparental mating with *E. coli* CM544 R751 (IncPβ plasmid) and *Pseudomonas putida* pSM1890 (gfp, Km, Gm, Sm) as donors (Peixoto et al., in preparation). *Pseudomonas putida* pSM1890 was provided by S. Molin (DTU, Denmark). The donor and recipient strains grew overnight in Müller-Hinton (MH)-broth supplemented with streptomycin (50 µg/ml) and

gentamycin (10 µg/ml) for *P. putida* pSM1890, with trimethoprim (20 µg/ml) for *E. coli* CM544 R751 and with rifampicin (50 µg/ml) for the recipient strains. Overnight cultures were centrifuged at 2250 *g* and the resulting pellets were resuspended in fresh Luria Bertani (LB)-broth. Two hundred µl each of the two donor strains and the recipient strain were mixed in a sterile Eppendorf tube and centrifuged for 5 min. The pellet was resuspended in 200 µl and transferred to a Millipore filter (0.22 µm) placed on MH-agar. After overnight incubation at 28°C the filters were removed and the cell lawns were resuspended with 0.85% sterile saline by vortexing, and serial dilutions were plated onto MH-agar supplemented with rifampicin (50 µg/ml), streptomycin (50 µg/ml) and gentamicin (10 µg/ml). The presence of the IncQ plasmid pSM1890 was confirmed by PCR as described by Götz et al. (1996). The *gfp*-labelled antagonists were re-tested for their ***in vitro*** antagonistic activity against *R. solanacearum* 1609 and strain identity was confirmed by BOX-PCR.

Inoculation of tomato plantlets with gfp-labelled antagonists

The rifampicin and *gfp*-labelled antagonists were grown overnight in LB-broth with rifampicin and gentamicin added. The roots of three-week old tomato plants were dipped in the cell suspensions of *gfp*-labelled antagonists (with an OD of approx. 0.8 to 1.1) for approx. 5 min and then planted in potting soil with 16 plants per treatment. Six different treatments were performed: inoculation with strains PRF116, PRD16, PRD 18, or PRD38, and a control without inoculation. The plants grew under greenhouse conditions at approx. 20°C and 16h/8h light/dark cycles.

Sampling and sample processing

Samples were taken 1, 5, 11 and 29 days after inoculation. Four plants were taken per treatment and each plant was processed separately. Three plants were used for determination of the cfu and for recovery of the bacterial pellet for DNA/RNA extraction (still in progress), and one plant for CLSM analysis. Plants were shaken vigorously to remove soil not tightly adhering to the roots. To determine the cfu counts of the inoculant in the rhizosphere, roots with tightly adhering soil were treated with sterile water in a Stomacher blender for 3 min at maximum speed. For CLSM the roots were washed with phosphate-buffered saline (PBS: 100 mM NaH_2PO_4, 100 mM Na_2HPO_4, 1,3 M NaCl in H_2O, pH 7.2-7.4) to remove soil particles from the root and then fixed in a 4% paraformaldehyde solution in PBS at 4°C overnight. For long-term storage the roots were subsequently placed in an ethanol-PBS-mixture (1:1) at -20°C.

Determination of the total aerobic and of the inoculant counts

Serial dilutions of the cell suspension obtained after Stomacher treatment were plated on King's B medium with cycloheximide (100 µg/ml) and with or without gentamicin (10 µg/ml) and rifampicin (50 µg/ml) added. The counts were determined after 24 hours' growth at 28°C.

Confocal laser scanning microscopy

CLSM analysis was done using a Leica TCS SP2 with AOBS (Acusto-Optical-Beam-Splitter) and Leica Confocal Software, version 2.5. The GFP excitation was done at 488 nm.

Results and Discussion

Labelling of antagonists

Fluorescent marker genes such as the *gfp* gene coding for the green fluorescence protein provide valuable tools to follow the colonisation of inoculants by *in situ* microscopic methods

(Jansson, 2003). The *gfp* gene can be introduced into the inoculant bacterium either by insertion in the chromosome (transposon-tagging) or on a plasmid. To minimise the probability that genes which are essential for the antagonistic activity are affected by the chromosomal insertion of the *gfp* gene, we decided for the strategy to introduce the gfp localised on the IncQ plasmid pSM1890. IncQ plasmids are relatively small broad-host range plasmids which have been isolated from a wide range of gram-negative bacteria (Rawlings and Tietze, 2000). Gfp is not expressed in *P. putida* pSM1890 due to a chromosomally inserted lac repressor (Haagensen et al., 2002) and thus only transconjugants which captured plasmid pSM1890 should show the green fluorescence. Plasmid pSM1890 was mobilised by IncP-β plasmid R751 into all four antagonists selected for labelling. The following numbers of transconjugants were obtained for the gram-negative strains: PRF116: 2.5 x 10^4 ; PRD 16: 3.3 x 10^4, PRD18: 2.3 x 10^4, and PRD38: 1.7 x 10^4. Although the gfp was expressed in all strains the brightest fluorescence was observed for strain PRF116. The biggest and brightest colonies were selected among the transconjugants of each antagonist. The presence of the IncQ plasmid pSM1890 was also confirmed by PCR using primers which target the IncQ *oriV* region. The selected *gfp*-labelled antagonists were tested also for their *in vitro* antagonistic activity. With the exception of strain PRD18 all *gfp*-labelled strains displayed a comparable antagonistic activity. However, the antagonistic activity of strain PRD18 was clearly reduced. Although the mechanism behind this dramatically reduced antagonistic activity is unclear, an interaction of plasmid-encoded function with the host background can be supposed.

Survival of the gfp-tagged recipients in the rhizosphere of tomato plants monitored by selective plating

The survival of the *gfp*-tagged antagonists in the rhizosphere of tomato plants grown under greenhouse conditions was followed by selective plating 1, 5, 11, 29 days after inoculation. Plating on King's B medium supplemented with rifampicin and gentamicin allowed a very specific detection of the inoculant since background growth was negligible for the rhizosphere of the non-inoculated control plants. All strains showed a good rhizosphere competence (see Fig. 1). The inoculant counts ranged between 7 x 10^7 up to 1 x 10^9 cfu/g root fresh weight at day 1 after inoculation. However, the inoculant counts varied between the single plants up to one order of magnitude (see Fig. 1).

During the time course of the greenhouse experiment the inoculant cfu numbers dropped and reached about 10^6 and 10^7 cfu/g root fresh weight 29 days after inoculation. The total cfu/g root fresh weight determined on King's B medium without gentamicin and rifampicin also ranged from 10^6 and 10^7. Plate counts on selective and non-selective media indicated that the IncQ plasmid pSM1890 was stably maintained in its hosts in the tomato rhizosphere.

In situ detection of gfp-labelled antagonists in the rhizosphere by confocal laser scanning microscopy

To obtain information on the colonisation patterns, in particular on the localisation of the inoculant strains on the roots, the root system of one tomato plant per treatment was analysed by CLSM 5 and 29 days after inoculation. Similar colonisation patterns were observed for all of the antagonists. Although no quantification was done it seemed that the best colonisation rates were detected for strain PRF116. Interestingly, this was also the strain which showed the strongest fluorescence. The fluorescence of strain PRD 38 was clearly lower than that of the other antagonists. Overall, the roots were quite well colonised. Closely colonised areas and weakly colonised regions were observed. No or only very few tagged bacteria were found on the root tips and on newly emerged side roots and on soil particles. Sometimes lots of GFP-tagged bacteria were found on the root hairs, and sometimes none at all.

Twenty-nine days after inoculation, in all cases only very few *gfp*-tagged cells were found, especially in deepenings, e.g., the borders of the root cells. The highest colonisation rate was still found for strain PRF116 followed by PRD16, and PRD18. No *gfp*-tagged cells could be detected on roots inoculated with PRD38. Remarkably, the cfu counts of PRD38 were approx. one order of magnitude higher than the counts of PRF116. Thus the *Enterobacter* strain PRF116 seems to be metabolically active for a longer time than the *Pseudomonas putida* strain PRD38.

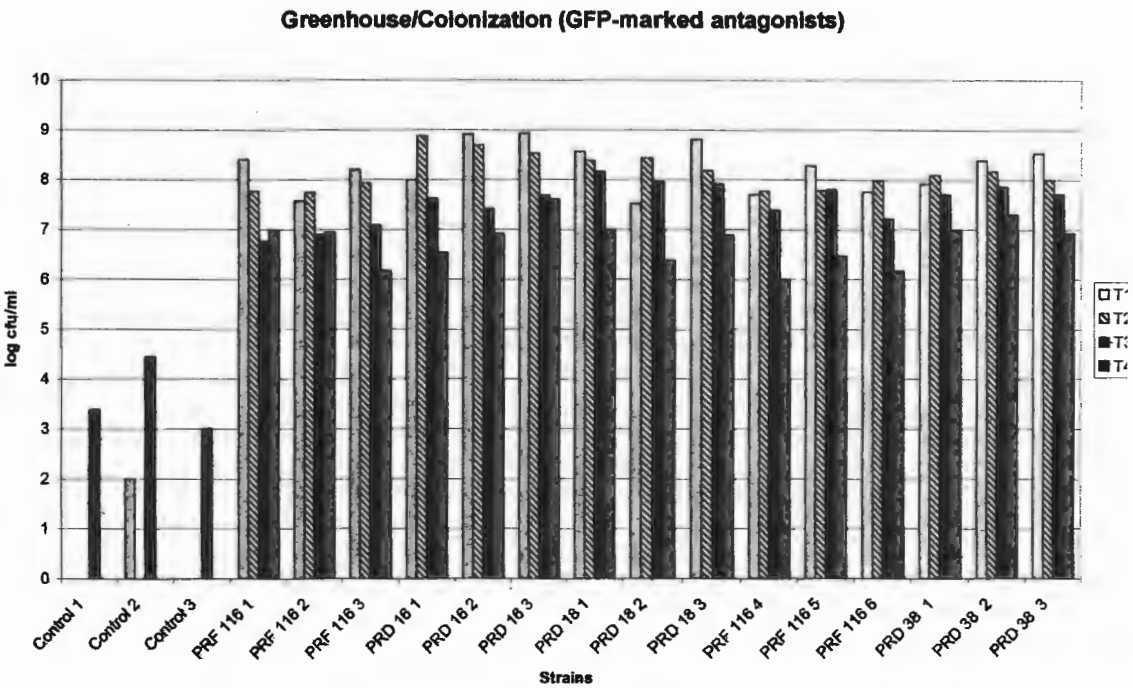

Figure 1. Survival of the antagonist in the tomato rhizosphere: Cfu counts on King's B supplemented with rifampicin and gentamicin; T1: 1 dpi, T2: 5 dpi, T3: 11 dpi; T4: 29 dpi

Conclusion

The results of the study indicate the need to combine both cultivation-dependent and -independent methods. While plate counts provide information on the number of cells present in the rhizosphere which are able to form colonies on solid medium it always remains unclear whether these cells are metabolically active and how they are distributed along the roots. CLSM allows to provide this kind of information. Under the inoculation and growth conditions used here, the *gfp*-tagged antagonists do not seem to colonise newly emerging roots. This kind of information on the localisation is also essential for future root sampling strategies. In addition, the effect of the inoculum on the bacterial and fungal rhizosphere community is presently monitored by DGGE analysis of 16S rDNA fragments amplified from directly extracted rhizosphere DNA.

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Pasteuria penetrans - friend, tease or distraction?

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Abstract: An environmentally safe alternative to chemicals *Pasteuria penetrans* can control root-knot nematodes and would make an ideal biological control agent. Why, after many years of research is *Pasteuria* an enigma? Recognition, epidemiology, host specificity and production are all perceived as drawbacks; nevertheless we maintain that *P. penetrans* can have a role in an IPM strategy.

Keywords: biological control, root-knot nematodes, *Meloidogyne* spp.

Introduction

An obligate parasite of root-knot nematodes, *Pasteuria penetrans* has received considerable attention as a biological control agent for this important group of crop pests (Chen & Dixon, 1998). Examples of soils where *P. penetrans* has induced a natural suppressiveness to root-knot nematodes are uncommon (Stirling & White, 1982) this may be due to the fact that such situations have not been recognised. *P. penetrans* is often found associated with root-knot but at low level of incidence Surveys in some countries find *P. penetrans* at 20-30% of sites sampled (Trudgill *et al.*, 2000). The ecological conditions favouring the parasite are not fully understood. The spores of *P. penetrans* cannot be readily estimated from soil samples and spores adhering to second stage juveniles recovered from soil are often un-noticed, unless observed at magnifications >X100. Similarly, *P. penetrans*-infected females may be overlooked when making routine assessments of infested roots as nematologists have no need to make observations at high power magnifications.

How can efficacy be improved?

Contriving suppressiveness is possible and in fields where *P. penetrans* was applied at 1000 spores per gram, the incidence of spore encumbered juveniles in soil and infected females in roots increased when a series of root-knot susceptible crops was grown (Trivino and Gowen 1966; Gowen *et al.*, 1998).

Different populations of *P. penetrans* may have specific host preferences, similarly, some root-knot nematodes within a field population may not be encumbered by spores of a particular *P. penetrans* population (Tzortzakakis and Gowen, 1994; Tzortzakakis *et al.*, 1996). When a population of *M. javanica* was repeatedly exposed to a single *P. penetrans* population (Pp3) a sub-population began to emerge which was not susceptible to that *Pasteuria*. Undoubtedly, this is a concern for anyone wishing to produce a commercial product of *P. penetrans*. Producing a population with a wider genetic base is an obvious strategy. This can be achieved by combining isolates of *P. penetrans* from different sources.

We believe that spore density is an important epidemiological consideration; if so, can sufficient spore populations be maintained to ensure that the majority of migrating juveniles acquire spores before invading a root? Further research is urgently needed to monitor the relationship of spore density with nematode density. In soils with high root-knot nematode densities greater numbers will escape contact and could lead to such intense infection (crop damage) that the presence of *P. penetrans* will be overlooked, possibly for several crop generations. Should the strategy be to wait for natural suppression to develop or intervene with a knock-down treatment? It is possible that high initial inundative applications of spores might be successful.

Further research is also needed to determine how quickly are spores "lost" from the system? We suspect that spore density declines as spores are carried to lower soil horizons. We know nothing about the ingestion of spores by soil micro-fauna.

The integrated use of *P. penetrans* with other biological control agents is a potential strategy – we see that combined use of *P. penetrans* spores and treatments with *Pochonia chlamydosporia* and possibly *Paecilomyces lilacinus* could give better control since *Pasteuria* infects at the development stage and the two fungi are egg pathogens (killing the eggs of females that have not been infected by *P. penetrans*).

Are there any known interactions? There is no reason to believe that *P. penetrans* spores have any influence on microbial soil or rhizosphere colonists, do these other microflora influence *P. penetrans*? Some work from Senegal suggests so (Duponnois *et al.*, 1997).

The use of biological control agents within a crop rotation of non-hosts could be an attractive option because if feasible, rotation will always be used in an IPM system. Non-host crops will help reduce nematode population levels but there is a risk in growing non-hosts that the spore populations will not persist in the rooting zones. The conflicting issues of growing non-hosts but wanting to sustain spore densities need further evaluation. Crop rotation with non-hosts is probably not an option if *P. penetrans* is to be used in intensive "protected" cropping systems where root-knot nematode susceptible crops are grown continuously.

In some tropical and sub-tropical farming systems the integration of use of *P. penetrans* with solarisation, soil desiccation, trash burning, or even nematicides is possible because of the relative indestructibility of *Pasteuria* spores.

Is spore production a constraint? We think not

All too often *Pasteuria's* potential as a biological control agent is maligned by the fact it cannot be produced *in vitro* and that we are still reliant on the *in vivo* production system first described by Stirling and Wachtel (1980). Oostendorp *et al.* (1991) state "However *Pasteuria penetrans* has not been grown successfully on artificial media. It must be reared on nematodes in the greenhouse or in nematode excised root systems. Neither procedure provides for the production of sufficient inoculum for large-scale application" Why should *in vivo* production be such an impediment? Many predators and parasitoids that are produced for the control of common glasshouse insect pests are produced *in vivo*. The implication that sufficient quantities of spores cannot be produced by this method is unfounded.

Spore production on a *per* female or *per* plant basis can be affected by many factors, host nematode, host plant, nematode density (Pembroke *et al.*, 1998). Stirling (1981) and Darban, pers. com. (2002) have shown that *Pasteuria* infected root-knot nematodes females can produce *circa* 2 million spores; therefore *P. penetrans* spore production should not be seen as a constraint.

Hypothetically

If you inoculate a 15 cm tomato plant growing in a 1-litre pot with 5000 J2 encumbered with 5 - 10 *P. penetrans* spores.

On the assumption that:

$^1/_3$ of juveniles invade = 1666.6
(60% of the $^1/_3$ are *P. penetrans* infected)
= 1000 infected females/plant

Potential spore production:

1000 infected females x 2 million spores per female
$= 2 \times 10^9$ spores/plant

You could treat:

2000 1 litre pots @ 1000 spores/g soil
200 1 litre pots @ 10,000 spores/g soil
120 microplots @ 1000 spores/g soil (microplot 100 x 100 x 10 cm)

Conclusion

We continue to be hampered in the promotion of *P. penetrans* as a means of managing root-knot nematodes. It has many of the attributes that would be attractive to commercial and organic producers specialising in cultivation of protected and high value field crops. Potentially it is so effective but we still lack some basic understanding on how it can be manipulated in these situations. Furthermore, recognition of the various stages of *P. penetrans* and its life cycle requires detailed study by experienced personnel. *In vivo* systems could generate sufficient quantities of spores for growers. Further research is needed to understand the relationship of both spore and nematode densities where species complexes may also exist. The final challenge is to convince the users of its potential.

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Pasteuria penetrans : a tritrophic interaction?

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Abstract: In a pot experiment it was found that female root-knot nematodes that were originally encumbered with spores of *Pasteuria penetrans* but had not become infected showed lower fecundity than those that had had no contact with *P. penetrans*. Different explanations for this phenomenon are discussed but it is suggested that the infection of root-knot nematodes by *P. penetrans* can have a greater effect on the host plants than that resulting from non-parasitized nematodes.

Key words Root-knot nematodes, *Meloidogyne javanica*, biological control.

Introduction

It has been shown that in experimental treatments in which *Pasteuria penetrans* has been applied, the numbers of eggs per egg mass laid by root-knot nematode females apparently uninfected by *Pasteuria* are less than in the untreated control treatments (Melki, 1995; Rao *et al.*, 1997; Rao and Gowen, 1998). This has interesting biological significance and epidemiological importance for the use of *P. penetrans* as a control agent. This paper presents an analysis of this hitherto unexplained phenomenon relating to the parasitism of root-knot nematodes by *P. penetrans*. That the number of eggs produced by healthy root-knot nematode females is affected by the presence of *Pasteuria*-infected nematodes in the same host plant.

Pasteuria can assist in the management of root-knot nematodes in two ways; the primary mechanism of control is the prevention of egg production by the female nematode; the second, if spore attachment to the juvenile in the soil is high (>15 spores per juvenile), its potential to infect a root system will be impaired by the spore burden (Davies *et al.*, 1988). The latter could be described as a "nematicidal effect" i.e. disabling the juvenile before it is able to enter its host. The evidence that fewer eggs are produced by healthy nematodes in plants where there are also *Pasteuria*-infected nematodes introduces a possible third mechanism of control and has an epidemiological significance as fewer juveniles will be produced by the second or subsequent generations.

Material and methods

The data evaluated in this paper comes from a pathogenicity study, comparing two populations of *Pasteuria penetrans* (Pp3) on two populations of *Meloidogyne javanica*, a single egg-mass line and the parent field population (Darban, 2003). There were four different *Pasteuria* treatments T1-T4 and two control treatments T5 and T6 (no *Pasteuria*). The interest in this data set was to analyse the relationship between the numbers of eggs per egg mass in the *Pasteuria* treatments compared to those in the control treatments. However though these results are the main focus of discussion it is necessary to relate them to the relative infection by *Pasteuria penetrans* in the females. For the purpose of this analysis comparisons are not

being made between the different *Pasteuria* treatments. Data were analysed by analysis of variance.

Four-week-old tomato plants growing in 500cm^3 in John Innes Number 2 compost were inoculated with 800 freshly hatched second stage juveniles encumbered with 6-10 spores per juvenile. Plants were maintained in a control temperature room at 25–28^{0}C with 16h daylight. Plants were evaluated after 30 and 45 calendar days (510 and 765 degree days accumulated at 17 degree days per calendar day). All treatments were replicated five times. Total numbers of females, egg masses, eggs per egg mass (mean of ten egg masses per replicate plant) and percentage infected females based on 20 females per replicate plant. The two harvest times enabled more accurate assessment of *Pasteuria* infection in the females.

Results and discussion

There were significantly fewer females in the *Pasteuria* treatments at first harvest compared to the control treatments, suggesting that juveniles that were spore encumbered were less able to invade, though the attachment levels of 6-10 spore per juvenile should not have impeded invasion (Davies *et al.*, 1988). An alternative explanation is that some juveniles in the *Pasteuria* treatments may not have developed into adults, this can only be speculated, as there was not a harvest time to assess invasion. However in the second harvest there were still significantly fewer females in the *Pasteuria* treatments, relating to the fact that fewer females had produced egg masses in the first harvest.

Table 1. The effects of *Pasteuria* infection on the numbers of *Meloidogyne javanica* females in root systems and their fecundity 30 and 45 days after inoculation of tomato plants with 800 spore-encumbered (T1-T4) or unencumbered juveniles (T5-T6).

	Total number of females		% infected females		Total number of egg masses		Number of eggs per egg mass	
Treatments	30 days	45 days	30 days	45 days	30 days	45 days	30 days	45 days
T1	126	247	50	34	69	104	119	302
T2	82	198	66	40	40	90	129	320
T3	61	153	69	54	24	62	121	317
T4	71	176	67	41	34	88	124	339
T5	162	326	-	-	118	224	229	422
T6	182	352	-	-	119	248	230	412
SED	6.78	21.11	3.7	3.3	7.74	10.33	8.62	8.62
P Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

In all treatments that were inoculated with encumbered juveniles, *Pasteuria* infection was present in the females, One might expect the percentage infection in the first harvest to have been higher, this could be explained by the fact that at an average temperature of 27^{0}C and with only 510 degree days accumulated, that not all infected females would have contained

mature spores (Stirling, 1981) and infection would have been based on the presence of early infective stages of the parasite which could have been overlooked. The decrease in the percentage of infected females in the second harvest can easily be explained by the presence of healthy second-generation females. This suggests that a harvest time between 30 and 40 calendar days at this temperature may reflect more accurately the number of infected females. Significantly more eggs were produced in egg masses in the control treatments, interestingly the same effect was observed in the second harvest.

Melki (1995) postulated the theory of latent infection, suggesting that infection by the parasite had slowed down the development of the female delaying egg production. His trials were done in field soils in a commercial plastic tunnel in Lebanon where *Pasteuria* was applied either in a spore-laden dry powder or in a water drench. Application by this method could mean that some of the nematodes that invaded the root system escaped attachment by *Pasteuria* spores signifying that not all females would become infected by *Pasteuria.* Therefore some of the egg masses assessed should have contained a "normal" complement of eggs, similar to those found in the control treatments.

Resistance to the disease implies *Pasteuria* may have triggered an immune response to the infection, which again would lead to a weakened nematode, either reducing or delaying egg production. This might happen where *Pasteuria* was introduced into the system by inoculating with pre-encumbered juveniles (i.e. 100% of all invading juveniles are known to be spore encumbered). This could, therefore, explain the reduced numbers of eggs per egg mass in the first harvest of this experiment where all juveniles had spores attached at inoculation (Table 1). Some of the females had produced egg masses at the time of the first harvest and all nematodes had been exposed to the parasite yet a number of these did not appear to produce infection stages of *Pasteuria*; reduced eggs per egg mass could be a symptom of exposure, latent infection or resistance. But this reasoning cannot explain either the field results from Lebanon (Melki, 1995) or from the second harvest of this experiment.

As discussed earlier, at the second harvest (45 days) there were many second-generation females, but the second stage juveniles that created them had not been exposed to *Pasteuria* spores. Consequently, it could be assumed that none of the second-generation females would be infected by *Pasteuria.*

Table1 and Figure 1 clearly show significantly fewer eggs per egg mass in the *Pasteuria* treatments in the second harvest compared to the control treatments. One might expect the second harvest in Figure 1 to show a range of numbers of eggs per egg mass with a cohort of females producing numbers of eggs similar to those of the control treatments, this is not the case. As stated, some females had not (as juveniles) been exposed to *Pasteuria* but have apparently been affected in such a way as to reduce fecundity. One could reason that nematode density pressure has induced stress in the host plant, which has resulted in reduced egg production, however comparing the total number of females between treatments in Table 1 there are on average, one third more females in the control treatments. This introduces an additional dilemma, it would be expected that as the number of egg masses increased, the number of eggs per egg mass might decline due to nematode density pressure on the host plant, or conversely one might expect more eggs in the *Pasteuria* treatments where fewer egg masses have been produced, this was not so.

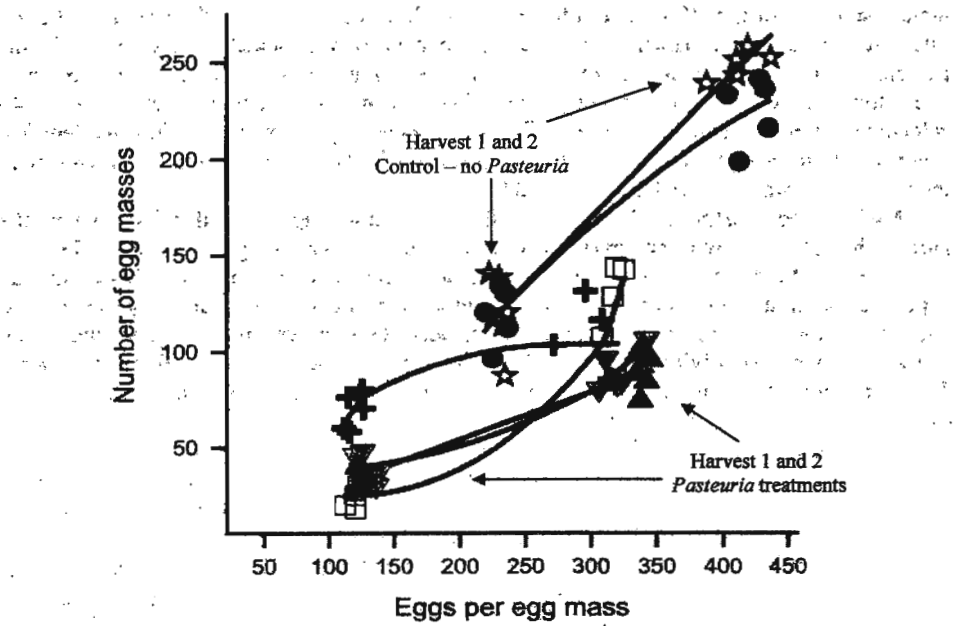

Figure 1 Relationship of numbers of egg masses to eggs per egg mass on tomato plants, 30 days (harvest 1) and 45 days (harvest 2) after inoculation with 800 spore-encumbered or unencumbered *Meloidogyne javanica* juveniles.

Melakeberhan and Ferris (1989) showed that the energy demand by *Meloidogyne incognita* on grape seedlings accounts for a significant portion of the total energy assimilated by the plant. This suggests that the energy demand by the nematode is a significant factor in the host-plant/nematode interaction. Pembroke *et al.* (1998) suggested that *P. penetrans* - infected nematodes might require more resources from the host plant than healthy nematodes. This theory was hypothesized because in experiments using different nematode densities with and without spore encumbered juveniles inoculated on two host crops, tomato and okra, it was observed that spore production on a per female basis declined significantly more at the higher nematode densities than the production of eggs per egg mass. The data has been reanalyzed and is presented here in Figure 2, original data taken from Giannakou (1998). This figure illustrates the marked decline in spores per female as the nematode density increases; there was no significant difference between numbers of eggs per egg mass at the different nematode densities.

In this discussion it should be made clear that the data presented in Figure 2 relates to the numbers of eggs per egg mass from control plants without *Pasteuria*, and the comparison is being made between spore and egg production at different nematode densities, initiated from juveniles with or without spores attached. Unfortunately this data set does not contain information relating to the total numbers of females or egg masses produced, the only factor relating to nematode density is from that of the original inoculum levels. Therefore it is difficult to discuss the inter-specific competition of the nematodes in this experiment However the data presented in Table 1 shows that where *Pasteuria* encumbered juveniles

were used as the inoculum there was a significant reduction in the total number of females in *Pasteuria* treatments compared to the control. Therefore one might deduce from the data shown in Figure 2 that there could be fewer females in the *Pasteuria* treatments; both experiments used juveniles with a similar spore burden. One could hypothesise that if there were fewer females in the root system then spore production per female would not decline so greatly.

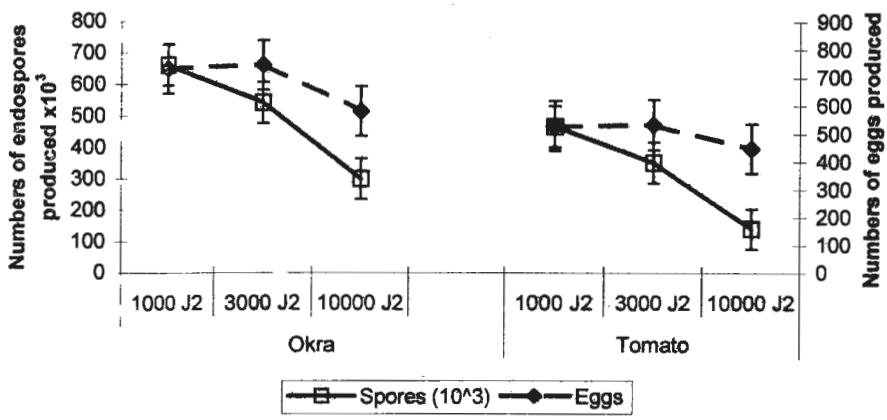

Figure 2. Relationship between numbers of spores per female or eggs per egg mass produced after 700 degree days (41 calendar) days at three inoculation densities of *Meloidogyne javanica* on two host crops inoculated with either spore encumbered (6-12 spores per juvenile) or unencumbered juveniles (adapted from Giannakou, 1998).

For reasons yet to be explained the presence of *Pasteuria* as part of a tri-trophic relationship is apparently affecting female fecundity in what should be healthy nematodes; yet under intensive production the hyperparasite produces a reduced number of spores on a per female basis. How does this knowledge affect the use of *Pasteuria* as a biological control agent? The suggestion that *Pasteuria* infected root-knot nematodes could be a greater drain on the host plant than non-infected nematodes is nothing but disturbing. Like most biological control systems, parasites or predators need the presence of the pest to maintain their own existence. It has always been understood that to induce a root-knot nematode suppressive soil with *P. penetrans* over several crops cycles it would be necessary to tolerate the nematodes to reproduce the *Pasteuria* – no pain no gain! It should also be remembered that *Pasteuria* does not kill the female, successful parasitism by *Pasteuria* eliminates egg production, thus reducing the potential number of juveniles produced in the second or subsequent generations; the on-cost however is that the female is producing spores instead.

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The effect of application of *Trichoderma viride* B35 (Pers. ex S.F. Grey) with iprodione on the rhizoplane microflora of *Allium porrum* (L.) and its infection with *Pyrenochaeta terrestris* ((Hansen) Gorenz, Walker et Larson)

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Abstract: Application of biocontrol agent *Trichoderma viride* B35 and fungicide iprodione used separately, as well as combination of both treatments, was studied under field conditions in two following vegetation seasons. The aim of the study was the evaluation of leek productivity, systemic resistance activity and rhizoplane microflora in soil naturally infested by *Pyrenochaeta terrestris*. Application of biocontrol agent into peat substrate, before seed sowing, resulted with significant growth stimulation of transplants. Moreover, increase of phenylalanine ammonia-lyase activity and slight increase of chitinase activity was found in tissue extracts from transplants after application of *T. viride* B35. Application of fungicide iprodione alone and combined with biocontrol treatments, as well as double application of *T. viride* B35 provided effective control of the pink root rot disease of leek caused *P. terrestris*. Noticeable decrease of the number of propagules of *P. terrestris* inhabiting root tissues and the "total number" of fungal propagules in the rhizoplane was observed after application of tested treatments. However, the "total number" of fungi inhabiting root tissues of plants, treated with tested agents, was only slightly lower in comparison with control plants. Among several groups of bacteria, enumerated in rhizoplane, a noticeable increase of *Pseudomonas* was observed after application of tested agents. The number of viable actionomycetes visibly increased after application of biocontrol agent alone and noticeable decreased after application of iprodione. The occurrence of viable spore-forming, proteolytic bacteria and bacteria grown on synthetic root-exudates did not change noticeably. We conclude that the elevated induced resistance as well as the shift of microbial communities might be the contributing factors, but not necessarily the main factors, in delaying disease development in leek and finally increase of marketable yield.

Key words: *Trichoderma,* biocontrol, leek, iprodione, integrated control, rhizoplane, microorganisms,

Introduction

The pink root rot disease of onion and leek caused by *Pyrenochaeta terrestris* became a sever problem in southern Poland in the past decade (Matkowski 1994). This pathogen could be controlled by soil fumigation (Porter *et al.* 1989), which is recognized as a method unfriendly to environment. Dipping of transplants of leek or onion sets in suspension of fungicides appeared to be an alternative method (Koriem *et al.* 1992, Biesiada & Kolota 2000). However, frequent use of chemicals in vegetable production could result in appearance of fungicides resistance races of pathogen.

The integration of chemical and biological methods of controlling of soil-borne pathogens seems to be a solution, which can overcame the problem of fungicide resistance of pathogens. It can decrease the fungicide input to environment also (Papvizas & Lewis 1988,

Elad & Stienberg 1994; De *et al.* 1996, O'Neill *et al.* 1996). The practical application of fungi from *Trichoderma* genus as biocontrol agents integrated with chemical treatments requires the selection of fungicide resistant isolates. We had selected several strains of *T. viride* resistant to benzymidazole and dicarboximide fungicides. Among them the strain B35 showed strong mycoparasitic activity and high activity of extra-cellular cell wall degrading enzymes (Pietr *et al.* 1993, Stankiewicz & Pietr 1998). In most cases, combined application of chemical soil disinfections with simultaneous application of *T. viride* B35 resulted with better efficacy of controlling of soil-borne pathogens of strawberry and vegetables in comparison with application of each treatment alone (Pietr *et al.* 2002, Slusarski & Pietr 2003).

Numerous reviews dealing with various aspect of biocontrol properties of *Trichoderma* (among others, Baker & Dickman 1993, Pietr 1997). Mycoparasitic activity, antibiotic production, plant growth stimulation, nutrient competition and induction of plant systemic resistance are discussed in literature factors explaining the biocontrol potential of *Trichoderma.*

The aim of the study was to determine the efficacy of controlling of pink root rot of leek using biocontrol agent applied alone and combined with fungicide iprodione. Moreover, the influence of tested methods on induction of systemic resistance and on the associations of rhizoplane microorganisms was studied.

Material and methods

Experiments with leek cultivar Arkansas were conducted at Horticultural Experimental Station of Agricultural University of Wroclaw under field conditions in two following vegetation seasons 1999 and 2000. Following chemical and biological treatments applied alone and combined together were tested: **Control** without any treatment. **Biocontrol agent** (*Trichoderma viride* strain B35) was applied as follows; **A** – biocontrol agent was applied before seed sowing (biomass of tested strain was mixed with peat substrate at a rate of 200 g m^{-3} (~10^9 c.f.u. per 1 g), **B** – biocontrol agent was applied at planting time (the root system of transplants were puddling in 2% water suspension of starch containing 50 g of *T. viride* per litre), and **A + B** both treatments were used. **Fungicide iprodione** (2% suspension of Rovral 255 SC e.g. 0.51% iprodione) was used at planting time. Transplants were dipped in fungicide suspension. In addition, combined applications of biocontrol agent with fungicide were tested. The field experiment was conducted as it was described earlier (Biesiada *et al.* 2002).

The influence of tested treatments on activity of enzymes involved in plant systemic resistance was determined by analyses of activity of PAL (Peltonen & Karjalainen 1995) and chitinase (Volpin *et al.* 1994). Samples of shoots of leek transplants were frozen in liquid nitrogen and stored at –70°C before analyses.

Six weeks after plantation the number of fungal propagules in the rhizoplane was enumerated on Martin medium ("total number") whereas of *T. viride* B35 on V8 medium (Papavizas & Lumsden 1982) with 100 ppm of iprodione. In addition, the number of viable cells of *Pseudomonas,* actinomycetes, spore-forming bacteria, proteolytic bacteria and bacteria able to grown on medium with synthetic root exudates, was determined by plate count (Biesiada *et al.* 2002). For this purpose, after washing out soil from the roots, the microorganisms from rhizoplane were washed out into 0.1 M $MgSO_4$ by ultrasonic treatment. The number of propagules of fungi on PDA („total number") and of *P. terrestris* on selective medium (Sneh *et al.* 1974) inhabiting roots tissues was determined during the harvest. After disinfection of root surface with 0.5% NaOCl (Biesiada *et al.* 2002) 180 roots segments (~5

mm) were placed on appropriate media. Samples of roots of 10 plants, randomly selected from each plot, were taken for analyses.

Results and discussion

Introduction of *T. viride* B35 into the peat substrate before seed sowing, revealed that it produced a well-pronounced increase of growth of leek transplants in the range 27.8% to 36.0% for roots and shoots respectively (Tab. 1). Moreover, significant increase of PAL activity in shoots of transplants was observed. The activity of chitinase was noticeable, however insignificantly, higher in comparison with activity in control plants (Tab. 1). The observed stimulating effects of *T. viride* B35 on the growth of leek suggest that the induction of systemic resistance occurs and it resulted in suppression of deleterious soil-borne microbes. Similar growth stimulation, in early stage of development of cabbage and tomato after application of *T. viride* B35 into potting substrate described Slusarski and Pietr (2003).

Table 1. The effect of application of *T. viride* B35 into peat substrate on the development of leek transplants and enzymatic activity in shoots before planting into the field (average from two seasons).

Treatment	Yield (dry matter – mg per plant)		Activity **	
	Roots	Shoots	PAL	Chitinase
Control	82.8 b *	239.0 b	4.779 b	1.023 a
T. viride B35	106.0 a	324.0 a	8.156 a	1.087 a

* - values in the same column followed by the same letter are not significantly different (t-Student test, P=0.05)

** - activity of PAL expressed in μg of trans-cynamonic acid / 1 g of tissue / 1 min.
- activity of chitinase expressed in μg of N-acetylglucosamine / 1 g of tissue / 1 min.

Table 2. The effect of biological and chemical treatments on the marketable yield of leek (t ha^{-1}) under field conditions (average from two seasons).

Chemical treatment	Biological treatment			
	Control	*T. viride* A	*T. viride* B	*T. viride* A + B
Control	13.62 c *	14.91 c	15.81 bc	19.21 a
Iprodine	18.66 ab	17.30 ab	N.D. **	17.98 ab

* - values followed by the same letter are not significantly different (t-Student test, P=0.05),
** - not determined,

Plant growth promotion of transplants resulted with visible yield improvement, but statistically insignificant in comparison with control (Tab. 2). Marketable yield of leek, after dipping of the transplants in the suspension of *T. viride* B35, was insignificantly different in comparison with control and also with similar treatment of transplants with fungicide suspension (Tab. 2). Double application of biocontrol agent, before seed sowing and again during planting time, revealed the highest increase of marketable yield of leek comparable to the effect of iprodione (Tab. 2). It is noticeable that combined application of fungicide and biocontrol agent did not result with synergistic effect. The final marketable yield of leek,

treated with iprodione, was similar independently from additional treatment of transplants with biocontrol agent (Tab. 2). However, applications of biocontrol agent *T. viride* B35, after soil disinfection with lower rate of dazomet (300 kg per ha), was in general superior to this chemical used alone at higher rates (400 kg per ha), as well as to single application of biocontrol agent under field conditions in production of cabbage, tomato and celeriac (Slusarski & Pietr, 2003) also as in production of strawberry runners and fruits (Pietr *et al.* 2002). These findings suggest that fumigation of soil create more favourable environment for introduced fungus than combined application of fungicide with biocontrol agent introduced only on root system of transplants.

The influence of tested treatments on microbial communities in the rhizoplane under field conditions was visibly variable depending on group of microbes and tested niche. The "total number" of fungi in rhizoplane, six weeks after planting, was significantly reduced after biological and chemical treatments (Tab. 3). Similar reduction of "total number" of fungi in rhizosphere of leek was described in our pervious paper (Biesiada *et al.* 2002). But the "total number" of fungi in rhizoplane during the harvest (data not shown) and isolated from internal space of roots was reduced only slightly (Tab. 4).

Double application of biocontrol agent and pudding of the transplants secure better colonization of roots than single incorporation into potting substrate only. Moreover, combined biological and chemical treatments of transplants improved slightly the rhizoplane colonization by introduced fungi. The number of propagules of *T. viride* B35 in rhizoplane was lower in 1998 and 1999 but was slightly higher in 2000 than in the rhizosphere of leek (Biesiada *et al.* 2002). These data suggest that the environmental conditions (temperature, soil moisture) influenced significantly the formation of propagation forms of fungi in soil.

Table 3. The effect of biological and chemical treatment on the presence some of microorganism in the rhizoplane of leek under field conditions 6 weeks after planting (average from two seasons).

Chemical treatment	Biological treatment			
	Control	*T. viride* A	*T. viride* B	*T. viride* A + B
	Fungi "total number" (c.f.u. per 1 g of roots)			
Control	2 990 a	1 560 b	1 470 b	1 040 b
Iprodine	1 070 b	1 360 b	N.D.	1 340 b
	T. viride B35 (c.f.u. per 1 g of roots)			
Control	0 d	250 c	550 b	700 ab
Iprodine	0 d	270 c	N.D.	950 a
	Pseudomonas (c.f.u. per 1 g of roots)			
Control	30 280 c	76 580 ab	66 840 b	66 050 b
Iprodine	96 070 a	67 610 ab	N.D.	92 450 a
	Actiomycetes (c.f.u. per 1 g of roots)			
Control	103 600 a	141 200 a	121 700 a	120 200 a
Iprodine	59 600 b	76 800 b	N.D.	59 900 b

* - values in the rows for each group of microorganisms followed by the same letter are not significantly different according to the t-Student test (t-Student test, P=0.05)

It is noticeable that application of biocontrol agent as well as of fungicide significantly increased the number of viable cells of *Pseudomonas* bacteria (Tab. 3). Moreover, the

application of *T. viride* B35 resulted with slight (~20%) increase and iprodione treatment significantly reduced the number of viable cells of actinomycetes (Tab. 3). Such effects were not observed in the rhizosphere (Biesiada *et al.* 2002). The bigger number of viable cells of *Pseudomonas* bacteria in the rhizoplane of leek can be taken under consideration as an additional factor suppressing the number of fungal propagules, including phytopathogenic *P. terrestris*. Inhibition of fungal microflora, after introduction of biocontrol agent, can also be connected with bigger number of viable cells of actinomycetes. Both groups of bacteria are well known antibiotic producers and several strains of *Pseudomonas* are very good root colonizers (among others Gottlieb *et al.* 2002).

Results of enumeration of viable cells of other groups of bacteria (spore-forming, proteolytic, bacteria grown on synthetic root-exudates) in rhizoplane of leek (date not shown) did not shown significant influence of applied treatments, similarly as was observed in the rhizosphere (Biesiada *et al.* 2002).

Table 4. The effect of biological and chemical treatment on the colonization of root tissues by fungi and *P. terrestris* under field conditions during harvest (average from two seasons).

Chemical treatment	Biological treatment			
	Control	*T. viride* A	*T. viride* B	*T. viride* A + B
	P. terrestris (c.f.u. per 1 cm of root segments)			
Control	0.40	N.D.	0.19	0.20
Iprodine	0.19	N.D.	N.D.	0.16
	Fungi "total number" (c.f.u. per 1 g of roots)			
Control	2.08	N.D.	1.87	1.82
Iprodine	1.96	N.D.	N.D.	1.99

Based on our studies we can assume that introduction of biocontrol agent *T. viride* B35 into leek rhizosphre elevated induced resistance, as well as resulted with the shift of microbial communities. These effects might be the contributing factors, but not necessarily main factors, in delaying of pink root rot disease development in leek. Observed suppression of development of phytopathogenic fungi resulted finally with increase of marketable yield of leek.

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Pathogen defense against biological control

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Abstract: Antibiotics produced by antagonistic bacteria play a key role in biological control of plant pathogenic fungi. Under field conditions, however, the ability of several antibiotic-producing strains to control plant pathogens has been inconsistent. This variability has been attributed to multiple factors, including inefficient colonisation of the host plant by the introduced bacterial strain, and temporal and spatial variation in the expression of antibiotic biosynthetic genes. Most of the factors studied with respect to improving the efficacy of biocontrol relate to improving the behaviour and activity of the biocontrol agent itself. In contrast, very little attention has been paid to the target pathogen and, more specifically, to defense mechanisms in the pathogen against biocontrol. Various mechanisms of natural resistance in target organisms have evolved to cope with antibiotics. In this study, the role of ATP-Binding Cassette (ABC) transporters in interactions between the plant pathogenic fungus *Botrytis cinerea* and antibiotic-producing *Pseudomonas* bacteria was investigated in detail. We discovered that phenazine antibiotics strongly induced expression of *BcatrB* from *B. cinerea*. ΔBcatrB mutants were significantly more sensitive to pure phenazine antibiotics than their parental strain, and *Pseudomonas* strains producing phenazines displayed a stronger antagonistic activity *in vitro* towards ΔBcatrB mutants than to the parental *B. cinerea* strain. On tomato leaves, phenazine-producing *Pseudomonas* strains were significantly more effective in reducing grey mould symptoms incited by a ΔBcatrB mutant than by the parental strain. We conclude that the ABC transporter BcatrB provides protection to *B. cinerea* in phenazine-mediated interactions with *Pseudomonas* spp.

Key words: antibiotics, *Pseudomonas*, *Botrytis*, ABC-transporters, microbial interactions

Introduction

Numerous *Pseudomonas* strains that produce antibiotics have been isolated from the rhizosphere of plants grown in soils from diverse geographical regions (Thomashow and Weller, 1996; Raaijmakers *et al.* 2002). The ability of plant-associated pseudomonads to produce antibiotics enables them to defend their habitats (Mazzola *et al.* 1992), whereas the existence of genotypic and physiological diversity among *Pseudomonas* spp. producing the same antibiotic (McSpadden-Gardener *et al.* 2000; Wang *et al.* 2001; Raaijmakers *et al.* 2002) provides a means by which populations of these microorganisms can adapt to diverse habitats. Although substantial progress has been made in the identification of genes involved in the biosynthesis and regulation of antibiotic production by *Pseudomonas* spp. (Chin-A-Woeng *et al.* 1998; Heeb and Haas, 2001), the responses of other microorganisms, and in particular defensive mechanisms, to these antibiotics have received little attention. Studies on the effect of therapeutic antibiotics, antimycotics and synthetic fungicides on human and plant pathogenic microorganisms have shown that target organisms can protect themselves by active efflux mechanisms that prevent the intracellular accumulation of these compounds to toxic concentrations (Higgins, 1992; De Waard, 1997). Active efflux mechanisms not only enable target organisms to tolerate exogenous antibiotics, but may also prevent self-

intoxication in antibiotic-producing microorganisms (Andrade *et al.* 2000). Among the active efflux mechanisms known, ATP-binding cassette (ABC) transporters are well studied (De Waard, 1997). The natural functions of ABC transporters from plant pathogenic fungi comprise secretion of toxins that act as virulence factors (host-specific toxins or mycotoxins) and protection against plant defence compounds (phytoalexins) and synthetic fungicides. It has frequently been postulated that ABC transporters may also play an important role in microbial interactions in natural environments. To date, however, their protective role in interactions between naturally occurring microorganisms has not been addressed. The objective of this study was to investigate whether fungal ABC transporters are involved in antibiotic-mediated interactions between bacteria and fungi in plant-associated environments. More specifically, we studied interactions between *Botrytis cinerea* and antibiotic-producing *Pseudomonas* spp. *in vitro* and *in situ*. For more extensive reviews on this topic we refer to Schoonbeek *et al.* (2002) and Duffy *et al.* (2003).

Results and Discussion

Expression of ABC transporter genes.
The effect of phenazine antibiotics on expression of ABC transporter genes *BcatrA, B, C, D, E, F, G, I,* and *K* in *B. cinerea* germlings was studied by northern analysis. The phenazine antibiotics phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) strongly induced *BcatrB* expression, whereas no effect was observed on the expression of *BcatrA, C, E, F, G, I,* and *K*. The induction patterns obtained after 60 min of treatment were similar to the patterns obtained after 20 min (data not shown). These results clearly demonstrate that phenazines produced by *Pseudomonas* spp. induced expression of a specific ABC transporter gene in *B. cinerea,* in particular *BcatrB*.

Antifungal activity of phenazine antibiotics.
Compounds that induce expression of ABC transporter genes can also be substrates of the encoded proteins. In that case, replacement of the gene involved may cause an increased sensitivity to these compounds. The activity of PCA and PCN was tested on radial mycelial growth of wild-type *B. cinerea* strain B05.10 and two independent ΔBcatrB mutants; a ΔBcatrA mutant was included as a control. Both ΔBcatrB mutants were significantly more sensitive to PCA and PCN than their parental strain B05.10 and the ΔBcatrA mutant. Spore germination experiments confirmed this differential sensitivity to these phenazines. Collectively, these results strongly suggest that the ABC transporter BcatrB in *B. cinerea* not only provides protection to the fungicide fludioxonil (Vermeulen *et al.* 2001) but also to the antibiotics PCA and PCN.

***Role of BcatrB in in vitro interactions between* B. cinerea *and* Pseudomonas.**
Antagonism of the *Pseudomonas* strains JM13, PCL1391, PCL1119 and Phz24 toward *B. cinerea* strains was determined in agar diffusion tests. JM13 does not produce any known antibiotics and showed almost no activity against all *B. cinerea* strains tested. Antagonistic activity of phenazine-producing strains PCL1391 and Phz24 was significantly stronger towards two ΔBcatrB mutants than to the parental strain B05.10 and ΔBcatrA. In contrast, PCL1119, the phenazine-deficient mutant of PCL1391, had almost no activity towards the ΔBcatrB mutants and their inhibition was similar to that of the control strains. Microscopic observation of germling growth in the inhibition zones confirmed that PCL1391 had a relatively strong effect on spore germination and germ tube elongation of the ΔBcatrB mutant B5. Similar observations were made in experiments with Phz24 and ΔBcatrB mutant B4. To

ascertain that the inhibition zones incited by PCL1391 and Phz24 can be, at least in part, ascribed to activity of phenazines, the concentration of these antibiotics in the agar plates was determined by HPLC analysis. Inhibition zones around inoculation sites of strain PCL1391 contained on average 30.1 $\mu g\ ml^{-1}$ PCN and zones around Phz24 contained 37.9 $\mu g\ ml^{-1}$ phenazines (32.5 $\mu g\ ml^{-1}$ 2'-OH-PCA and 5.4 $\mu g\ ml^{-1}$ PCA). These concentrations are approximately 2 times higher than the EC_{50} value of PCN for the ΔBcatrB mutants and 1.5 times lower than the EC_{50} value for the parental strain B05.10. In zones around the inoculation site of the other bacterial strains, including PCL1119, no phenazines were detected.

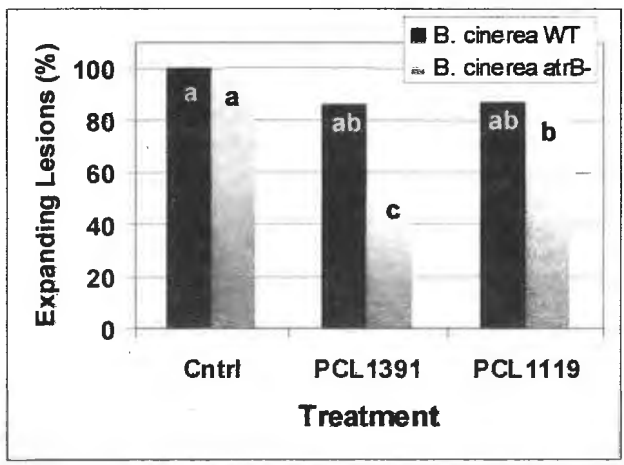

Figure 1: Antagonistic activity of *Pseudomonas chlororaphis* PCL1391 and its phenazine-deficient mutant PCL1119 on disease development incited by *Botrytis cinerea* strains B05.10 (black) and ΔBcatrB mutant B4 (grey) on tomato leaves. Leaves were sprayed with sodium phosphate buffer (control, cntrl), a suspension of PCL1391 or PCL1119 in the same phosphate buffer. Disease development is expressed as the percentage of *B. cinerea* inoculation sites developing into expanding lesions.

***Role of BcatrB in in situ interactions between* B. cinerea *and* Pseudomonas.**

The interactions between phenazine-producing *Pseudomonas* strains and *B. cinerea* strains B05.10 and ΔBcatrB4 was studied on tomato leaves. The virulence of B05.10 and ΔBcatrB4 on tomato leaves was similar in the control treatment. Treatment of tomato leaves with cell suspensions of PCL1391 significantly reduced the percentage of expanding lesions caused by ΔBcatrB4, whereas no reduction was observed for parental strain B05.10 (Fig. 1). PCL1119, the phenazine-deficient mutant of PCL1391, did not reduce the percentage of expanding lesions for both ΔBcatrB4 and B05.10. Furthermore, the effect of PCL1391 on the size and growth of the lesions caused by ΔBcatrB4 was significantly stronger than for B05.10. In contrast, the effect of PCL1119 on lesion size and growth was similar for B05.10 and ΔBcatrB4. In the repeat experiment, similar results were obtained: PCL1391 and also phenazine-producer Phz24 significantly reduced the size and growth of the lesions caused by ΔBcatrB4, whereas no reduction was observed for parental strain B05.10. HPLC analysis demonstrated that on leaves treated with PCL1391, PCN was produced at an average concentration of 5.6 $\mu g\ g^{-1}$ leaf fresh weight. No phenazines were detected on untreated leaves

or on leaves treated with PCL1119. These results indicate that the ABC transporter BcatrB provides protection to *B. cinerea* in phenazine-mediated interactions with *Pseudomonas* spp.

In conclusion, the results presented here and reviewed in more detail by Schoonbeek *et al.* (2002) demonstrate, for the first time, that fungal ABC transporters can play an important role in microbial interactions in plant-associated environments and, more specifically, protect a fungal pathogen against antibiotic compounds produced by antagonistic bacteria. Current work focuses on other protective mechanisms in *Botrytis cinerea* and *Fusarium oxysporum* against antibiotics produced by *Pseudomonas* species.

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Biocontrol of plant-parasitic nematodes by *Trichoderma harzianum*

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I. Possible role of proteases

Introduction

The fungal biocontrol agent *Trichoderma harzianum* is used against various fungal plant diseases and is active also against plant parasitic nematodes. Studies of the modes of action against the root-knot nematode *Meloidogyne javanica* showed that the strains IMI206040 (designated WT) and its transgenic strain P-2 which contains multiple copies of the 31 kDa proteinase gene *prb1*, presented the ability to attack the nematode eggs and juveniles (J2). Strain T-203(Y) presented such activity as well. The P-2 showed improved activity on the J2 as compared to the wild type (WT) strain. In greenhouse experiments, P-2 revealed improved nematicidal activity and extracts from *Trichoderma* (P-2) treated soil presented higher proteolytic activity as well as higher *in vitro* nematicidal activity Sharon et al., 2001).

Objectives

To study the role of proteases in biocontrol modes of action of the fungus *Trichoderma harzianum* against the root-knot nematode *Meloidogyne javanica.*

1. To determine the *in vitro* parasitic ability of various *Trichoderma* strains on root-knot nematode juveniles and eggs.
2. To monitor the proteinase *prb1* gene expression during the fungus-nematode interactions.
3. To test and characterize the nematicidal activity of various fungal strains culture filtrates against the root-knot nematode in comparison with other nematodes.
4. To detect proteolytic enzymes activity in fungal culture filtrates and the possible correlation with the filtrates nematicidal activity.

Materials and Methods

Effect of* Trichoderma harzianum *culture filtrates on* Meloidogyne javanica *J2
Fungal strains were cultured on potato dextrose broth (PDB), filtrated through 0.2μm membrane and subjected to *in vitro* assay in 96-well plates containing *M. javanica* J2 from axenic tomato roots cultures.

Direct interactions between the fungus and* Meloidogyne javanica *J2 and eggs:
Nematode eggs and J2 from axenic cultures were placed together with fungal spores suspension on a 10-fold diluted PDA medium, in chamber slides. For proteinase expression, a transgenic strain of IMI206040 carrying a fusion of the *prb1* promoter with the green fluorescent protein (GFP) gene was used.

Characterization of culture filtrate nematicidal activity on* Meloidogyne javanica *J2:
1. Filtrates were boiled for 10 min prior to the bioassay with J2. **2.** Filtrates were fractionated using a 3-kDa molecular-cutoff membrane and tested for nematicidal activity in 96 well plates, in sterile conditions.

Characterization of protease activity of fungal culture filtrates on zymograms:
Proteins were separated by electrophoresis on polyacrylamide gels (12%) containing casein. The gels were stained with Coomassie Brilliant Blue after incubation with developing buffer.

Results

Several *Trichoderma harzianum* strains were tested for their *in vitro* parasitism ability on *Meloidogyne javanica* 2nd stage juveniles (J2) and eggs. All the strains tested: IMI206040 (designated WT) and its transgenic strain P-2 which contains multiple copies of the proteinase gene *prb1*, strains T-203(Y), T-44, T-35 and T-315 were able to colonize J2 and pre-mature eggs (Fig 1). P-2 was the most effective one on J2. The fungal growth was enhanced in the presence of the nematode (Fig 2).

Culture filtrates of both WT and P-2 paralyzed and killed *M. javanica* J2 within 24-48 h, as well as other plant-parasitic nematodes, but had almost no effect not on beneficial insect parasitic nematodes (Table 1). The efficacy of the P-2 filtrate was higher than the WT, immobilizing the J2 within a shorter period. Filtrates from T-203, T-44 **did not** affect the J2 viability.

The filtrate activity on *M. javanica* J2 was heat sensitive and restricted to the low molecular weight fraction (less than 3-kDa). The higher molecular weight fraction, which contained the proteases, did not affect the J2. A ladder of proteolytic activity between 20-31 kDa, was detected on zymograms, **only** in WT and P-2 filtrates, which presented nematicidal activity in the low molecular weight fraction (Fig 4).

Conclusions

Various *Trichoderma harzianum* strains presented parasitic activity on *Meloidogyne javanica* eggs and juveniles. The transgenic strain P-2, which produces higher levels of the inductive proteinase prb1, had presented an improved activity on J2, as compared to the WT strain. Observation on *prb1* gene expression, using a GFP transformant, showed that this gene expression is enhanced during the fungus-nematode interaction. These results suggest the involvement of proteinase prb1 in the direct parasitism process.

Culture filtrates of the fungal WT and P-2 presented nematicidal activity against several plant-parasitic nematodes. Characterization of the nematicidal activity on *Meloidogyne javanica* juveniles revealed a positive correlation between the presence of a new ladder of proteases (20-31kDa) and production of heat sensitive, low molecular weight nematicidal compound/s in culture filtrates of WT and P-2. These results suggest an indirect role of proteases in the nematicidal activity of fungal culture filtrates on the *Meloidogyne javanica* J2.

II. Attachment of the fungus *Trichoderma harzianum* to the root-knot nematode *Meloidogyne javanica* leads to improve biocontrol activity

Direct fungal parasitism, is one of various possible mechanisms by which the fungus can act against the nematode juveniles (J2) and eggs. The fungal hyphae coil around the J2 and penetrate them, as well as to nematode eggs. Microscopic observations showed that the fungal spores, normally, do not attach to the nematodes, while the germinating hyphae attach and penetrate the nematodes. Antibodies raised against root-knot nematodes, that bind to the surface of *M. javanica* J2 and eggs, bound also to the fungal spores and agglutinated them. The presence of these antibodies enabled the attachment of the spores to the nematodes. This resulted in a significant enhancement of fungal parasitism on the nematodes. Moreover, the binding of antibodies to the spores enhanced their germination and thus improved the fungal parasitism on the nematode. The nature of the antibody binding to both nematodes and spores is currently under investigation. Use of antibodies or similar attachment mechanisms may lead to improved biocontrol of plant-parasitic nematodes by *Trichoderma.*

Strains of the fungal biocontrol agent *Tricoderma harzianum* can affect the root-knot nematode *Meloidogyne javanica* by various modes: 1. Direct parasitism on second-stage juveniles (J2) and eggs; 2. Metabolites produced by the fungus affect the J2 viability and egg hatching. *In vitro* parasitism ability was presented, to different extents, by all the strains tested: IMI206040 (designated WT) and its transgenic strain P-2 which contains multiple copies of the proteinase gene *prb1*, strains T-203(Y), T-44, T-35 and T-315. A transgenic strain of WT with the green fluorescent protein (GFP) gene attached to the *prb1* promoter, was used to demonstrate the involvement of this proteinase in the interaction between the fungus and the nematode. Culture filtrates of WT and P-2 paralyzed and killed the *M. javanica* J2, as well as other plant-parasitic nematodes. The activity on *M. javanica* J2 was heat sensitive and restricted to the low molecular weight fraction (less than 3 kDa). The higher molecular weight fraction, which contained the proteases, did not directly affect the J2. However, a ladder of proteases between 20-31 KDa, which presented proteolytic activity only at low pH range, was positively correlated to the nematicidic activity of the various strains filtrates.

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Suppressive soils, the edge of chaos and Multitrophic strategies for biocontrol of pests and diseases in soil ecosystems

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Key words: suppressive soil, antagonistic potential, root-knot nematodes, biocontrol

Suppressive soils & antagonistic potential

A *suppressive soil* is defined as a soil in which disease development in a susceptible host is suppressed in the presence of the pathogen or parasite. It is an umbrella term encompassing fungistasis, competitive saprophytic ability and other interactions where reduced disease occurs. However, the absence of a disease or pest is not justification to imply that a suppressive mechanism is involved (Schneider, 1982). Many other definitions of a suppressive soil exist but they all have one thing in common - they define the highest level of antagonistic potential that can exist in a soil to a specific pest or disease. Sikora (1992) defined the term *antagonistic potential* as the capacity of a soil ecosystem through biotic factors, to prevent or reduce the spread of a pathogen, parasite or other deleterious agent. Important, therefore, is the interrelationship of multiple factors both biotic and abiotic in a suppressive environment.

Every gram of soil has a measurable level of antagonistic activity toward a specific pest or disease, and all soil microorganisms, regardless of whether they are deleterious or beneficial, experience a degree of antagonistic activity: it is survival of the fittest on a microscopic scale. The level of antagonistic potential can range from extremely low to total suppressive (Baker & Cook, 1982). Therefore, biocontrol is the rule in agricultural soils and not the exception. However, the amount of yield loss that occurs in crops having a significant level of antagonistic activity is often still too high and thus the need for pesticides (Sikora 2002).

Individual organisms isolated out of the multitrophic structured antagonistic potential have been used for the past five decades in attempts to develop inundative systems of biological control. Pathologists due to mandate, funding or manpower availability are often forced to take a very focused approach to biocontrol and therefore concentrate on one target pest or disease and one antagonist. This approach has yielded only limited success and has stymied the widespread use of biological control in pest and disease management. This leads us to ask the following questions:

- If suppressive soils exist, why are we unable to use inundative approaches for biocontrol?
- Why has it been impossible to re-establish suppressiveness with antagonists isolated from such soils?
- What aspects have we misinterpreted in our present inundative approach that has prevented us from reaching our target?
- What antagonists are we overlooking or what aspects of application are we failing to adapt to our biological systems for success?

The balance of nature and organic amendments

Pimm (1991) stated that the general understanding of the *balance of nature* is based upon nature's own ability to restore itself after a major disturbance. It is often considered an attribute that comes from within nature. This healing process is an ecological process between populations of organisms and is connected to interactions between species in a community and between the community and the abiotic environment. For some, the balance of nature has mystical connotations. The use of organic amendments to stimulate antagonistic potential in soil is a tool used to re-establish the balance of nature in degraded agricultural soil. The suppressiveness attained is the result of high performance levels of the different antagonists in the soil ecosystem. If the end result is due to the action of a multitude of microorganisms acting in concert, then a solution to biocontrol may be to alter the single organism inundative approach. In plant pathology ecological concepts seem to be based on simple equilibria in nature and that the species behaviour in ecosystems is predicable. In actuality all species, and in particular species in the soil ecosystem, are part of a complex and dynamic system in which they may or may not interact with each other. Most organisms in the soil ecosystem, even if present in large numbers, seldom interact with each other – most of them are trying to survive or in laymen's terms – they prefer to be left alone.

The edge of chaos

The concept of *The Edge of Chaos* is a fascinating and highly relevant to suppressive soils in agro-ecosystems and to the development of biological control concepts. The concept states that ecosystems move through activity states, sometimes frozen, often chaotic, but they eventually come to rest, with fitness optimised or poised at the Edge of Chaos. It was once thought that individuals within a species or species in a community might shape their behaviour for the good of the group. Biologists now believe that individuals act in narrow, Darwinian, selfish ways and will cheat if they can to optimise themselves (Lewin 1992). Therefore we should visualize an ecosystem with many species interacting, each pursuing its own evolutionary ends, each evolutionarily tuning its own genetic connections and its interactions with other species, the result of which is that the community moves to a position of maximum sustained fitness or moves to the Edge of Chaos. This is a state in which an established microbial community can resist invasion by alien species, and is in essence the definition of a suppressive soil and the basis of biological control. Chaos is probably what occurs following the incorporation of organic amendments into soil. The community explodes in numbers and each individual species through individualistic self-serving activity reaches a high level of fitness. The result of which is suppression of a pests and/or diseases.

Therefore, one biological control strategy that should be considered in the future seems to be the establishment of some form of The Edge of Chaos in or around a plant through multitrophic inundative application of select antagonists that gives the plant a broad based antagonistic edge over its enemies. Such a strategy of course raises a number of questions:

1. Can we produce something resembling suppressive soils in commercial crop production without treating the entire field?
2. Can a predictable level of The Edge of Chaos be established for pest or disease biocontrol using a minimum number of antagonists?
3. Can multitrophic inundative systems, applied in the seedling stage, be used for effective biological control in an infested field?
4. Can we select antagonists that have individually optimised their competitive niche in the ecosystem and combined them effectively to work synergistically under field conditions?
5. Can a multitrophic approach be developed that is economical on a practical scale?

We believe an attempt must be made to place a wedge between the pest and the root system using multiple antagonists working independently and at times synergistically to achieve a biologically enhanced environment around young susceptible plants before transplanting into an infested field. This could be a successful approach to biocontrol when based on sound scientific data and economic analysis. The biological system management approach must target the immediate environment of the crop in the seedling stage and not just the target pest. This means that:

1. The microbial antagonists must be at their optimum level of fitness immediately following application
2. Antagonistic activity must be fast-acting and sustainable during that part of the infection process responsible for yield loss
3. Synergistic interactions between antagonists must be utilized
4. Biocontrol of the pest must sustain the costs of multitrophic inoculation
5. Application costs must be low and yield increased to cover application costs

Multitrophic biocontrol models

Few attempts have been made to develop multitrophic approaches to biocontrol. The impression, true or false, that multiple inoculations increase costs to unacceptable levels also has limited research in this area. A successful multitrophic model must be economically acceptable to the grower. We have developed a model that stresses biological enhancement of vegetable planting material.

An important factor in our decision to target seedling production systems is the reduced inoculum required as a major cost factor. At the same time, bacterial and fungal antagonists that are rhizosphere competent or that grow endophytically have been selected for the model system, because they are given a head start over niche competitors that are encountered in direct application to field soil. Since yield is often related to seedling health in the early stages of growth, an additional treatment at transplanting is being considered. Attempts also are being made to find antagonists that promote each other's fitness. In table 1, effective antagonists that could be combined at sowing and transplanting are listed.

Table 1. Suggested antagonists for the development of multitrophic models to reduce the impact of the root-knot nematode, *Meloidogyne* spp., by biologically enhancing planting material at sowing and transplanting.

Seedling treatment		**Transplant treatment**	
Antagonist Group	***Organisms***	***Antagonist Group***	***Organisms***
Arbuscular mycorrhiza	*Glomus intraradices* *Glomus* spp.	PHPR - plant health promoting rhizobacteria	*Rhizobium etl G12* *Bacillus sphaericus B43*
Mutualistic fungal endophytes	*Fusarium oxysporum 162* *Trichoderma atroviride*	trapping fungi	*Arthrobotrys superba* *Monacrosporum lysipagum*
Egg pathogenic fungi	*Paecilomyces lilacinus*	Egg pathogenic fungi	*Paecilomyces lilacinus* *Trichoderma harzianum*
Rhizo-competent egg pathogens	*Pochonia chlamydosporia*	Rhizo-competent egg pathogens	*Pochonia chlamydosporia*
		Endoparasitic fungi	*Hirsutella rhossiliensis* *Drechmeria coniospora*
		Obligate parasite*	*Pasteuria penetrans**

(*Added at transplanting to promote population build-up on low populations of root-knot following biocontrol)

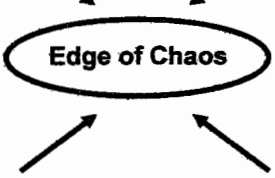
We are presently developing a multitrophic biocontrol system model targeted at biologically enhancing tomato planting material in the tropics, because suitable antagonists for this production system already exist. However, there are a number of other high value crops grown in seedling production systems, some from tissue culture, that also lend themselves well to a multitrophic approach for example: banana, pineapple, ornamental flowers and other vegetable crops. The proposed system presented here is based on the use of plant health promoting rhizobacteria (PHPR), arbuscular mycorrhizal fungi (AMF) and mycorrhizal promoting bacteria (MPB) or mutualistic endophytic fungi (MEF) to control the root knot nematode, *Meloidogyne incognita*, and to a lesser extent root pathogens on tomato. This model was selected for the following reasons:

1. PHPR reduce root-knot penetration
2. PHPR induced resistance to sedentary nematodes
3. PHPR also reduce fungal pathogen infestations
4. AMF and MEF are known to reduce root-knot penetration and reproduction
5. AMF and MEF reduce root pathogen infection
6. AMF increase phosphate uptake and offset root damage at transplanting
7. MHB increase AMF colonization
8. MHB have antagonistic activity toward root-knot and pathogens

Our strategy is based on reducing early root penetration of the nematode in tomato production where seedling transplant technology is the norm. The main thrust of our research is to find plant health promoting rhizobacteria antagonistic toward root-knot nematodes (PHPR) with simultaneous mycorrhizal promoting activity (MPB) or PHPR that work synergistically with mutualistic endophytic fungi (MEF). Literature for each antagonist is given in figure 1.

PHPR against nematodes	
Zavaletta-Mejia & van Gundy	1982
Becker et al.	1988
Oostendorp & Sikora	1990
Stirling et al.	1991
Terhardt et al.	1997
Hasky Günther et al.	1998
Reitz et al.	2000
Hallmann	2001
Reimann & Sikora	2003

AMF against nematodes	
Sikora & Schönbeck	1975
Bagyaraj et al.	1984
Saleh & Sikora	1984
Smith	1987
Rao et al.	1995
Jaizme-Vega et al.	1997
Nagesh et al.	1999
Waceke et al.	2001
Talavera et al.	2002



Mutualistic Endophytic Fungi (MEF)	
Hallmann et al.	1994
Hallmann & Sikora	1996
Niere et al.	1999
Sikora et al.	2003

Mycorrhiza Promoting Bacteria (MPB)	
von Alten et al.	1993
Toro et al.	1997
Mamatha et al.	2002
Duponnois & Plenchette	2003

Figure 1. Literature on biocontrol of root-knot nematodes with Plant Health-Promoting Rhizobacteria, Mutualistic Endophytic Fungi, Arbuscular Mycorrhizal Fungi and Mycorrhizal Promoting Bacteria.

We are attempting to utilize a segment of "The Edge of Chaos" for biological control, by selecting organisms that are known nematode antagonists and by applying them in a manner that increases their chances of reaching high levels of fitness and biocontrol in the rhizosphere. By using the transplanting production system we are hoping to neutralize the soil ecosystem to the advantage of the antagonists by eliminating competing microbes.

Outlook

Past research has demonstrated that single and multitrophic inoculations with PHPR, MEF, or AMF with or without MPB can lead to a reduction in nematode infection and population development (Sikora 1992, Hallmann et al 1994, Hasky-Günther et al. 1989, Niere et al 1999, Reimann & Sikora 2003). Many of these organisms also simultaneously affect plant pathogens. Therefore, we believe the concept of biologically enhancing seedlings can lead to effective plant protection toward nematode and pathogen infection. It also is axiomatic that treatment of the seedlings with biological control agents will reduce costs on a per ha basis significantly over field treatment with nematicides or inundative application of similar antagonists. The difference is application to approximately 10,000 seedlings or seeds versus 2500 tons of soils. In addition, the use of plant growth promoting microorganisms like AMF simultaneously increases plant survival and nutritional aspects.

How these synergistic interactions will affect the nematode over the growing season is being studied. The biological management strategy we envision is shown in Figure 2. The model is based on former knowledge and the following premises:

1. MEF or AMF will establish well in the seedling units due to lack of competition
2. MPB will further increase AMF and promote control of root-knot after transplanting
3. At transplanting PHPR isolates applied to the seedlings will induce resistance and protect the root during this sensitive early establishment growth period
4. Extensive MEF or AMF colonization of the root attained in the seedlings stage will lead to long-term biocontrol after transplantation to the field

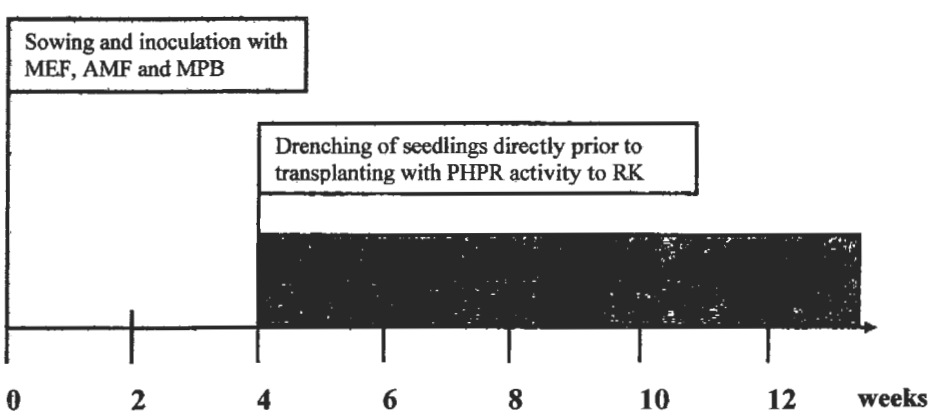


Figure 2. Biological system management strategy using a multitrophic package of antagonists of PHPR, MEF, AMF and MPB to reduced root-knot nematode, *Meloidogyne* spp., early root penetration and reproduction on tomato.

With this system we expect:

1) Significant increase in MEF colonization of root system
2) Increase in AMF colonization due to MPB
3) Short-term nematode reduction through PHPR induced resistance
4) Long-term suppression of nematode growth and reproduction by MEF or AMF
5) Supplementary control of secondary root pathogens and
6) Increased plant establishment and growth due to AMF

We believe this multitrophic form of biological system management will lead to significant reductions in root infections due to nematodes but also pathogens not only during the early root infection process but also give control activity throughout the growing season. This is one approach to developing a multitrophic approach to integrated control. Other combinations of antagonists need to be analysed for their effects on biological enhancement of planting material taking into consideration both crop plant, nematode or pathogen and crop production attributes (Tab. 1). However, we believe this is a logical first model for nematode management and a good start in the direction of "The Edge of Chaos".

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Multitrophic Interactions in Soil and Integrated Control
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Impact of organic amendments on soil suppressiveness to diseases

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Abstract : The ability of spent mushroom compost and cattle manure to improve soil suppressiveness both to Fusarium wilt and to *R. solani* diseases was evaluated in two soils in relation to alterations of the indigenous microbial communities. Beneficial effects of the organic amendments on disease suppression were observed in both soils. The organic matter stimulated the community level physiological activity of the bacteria, and provoked changes in the structure of the fungal communities. The intensity of the bacterial activities was mainly associated to the soil suppressiveness to Fusarium wilt but had no interaction with soil suppressiveness to *R. solani* diseases. The changes in the composition of the fungal communities could be associated to emerging specific populations able to antagonize *R. solani* and/or *F. oxysporum*. Because organic amendments could enhanced soil suppressiveness to diseases, they appeared as a potential management practice to control Fusarium wilt and *R. solani* damping-off.

Key words : *Fusarium oxysporum, Rhizoctonia solani*, compost, manure, bioassay, bacterial activity, Biolog, fungal community structure, T-RFLP.

Introduction

Soils suppressive to diseases induced by the most important soil-borne pathogens have been described in many areas; they include fungal and bacterial pathogens but also nematodes and they control root rot and wilt diseases induced by many different species (Alabouvette et al 1996). Every soil has some potential of disease suppression, leading to the concept of soil receptivity to a disease, that is its capacity to control the pathogenic activity. Various hypotheses have been proposed to explain mechanisms limiting the pathogenic activity in suppressive soils. They come within either biotic factors or abiotic factors or both (Höper and Alabouvette 1996). Each of the mechanisms proposed is relevant to a specific case study and cannot explain similarly all the situations encountered. Because of the physico-chemical properties of the soil and the biodiversity of the microbial community it harbors, the soil interferes in several ways with the relationships between and among micro-organisms, pathogens and plants, and it can modify the interactions among micro-organisms themselves (Abawi and Widmer, 2000). Taking into account the suppressiveness of soils to diseases caused by two pathogens having different ecological requirements, we want to illustrate the diversity of mechanisms responsible for disease suppression, and to show the possibility to promote the soil health by altering cultural practices and their subsequent effects on the components and the activity of the soil biota. The addition of organic amendments into soils should exemplify some of the mechanisms involved in soil suppressiveness to diseases.

Materials and Methods

Soils and Organic amendments

Two types of soils, Dijon and Ouroux were chosen as intermediate suppressive and conducive soils respectively to Fusarium wilt and vice versa to *Rhizoctonia solani* diseases. Dijon was an alkaline clayey soil with high content of organic C while Ouroux was a sandy soil having low organic C. These soils were dried, sieved (4 mm) and kept in plastic bins before use. When needed, sterile soil was obtained by autoclaving soil 3 times for 1 hour at 105°C with an interval of 24h..

Two organic materials (compost and manure) were added to these soils as amendments at varying incorporation rate (0, 5, 15 and 30 % v/v). Compost derived from spent mushroom substrate was used fresh. Manure derived from dairy cattle was dried and grounded by the manufacturer (SERP, Dijon). Unamended soils were used as control. The soil and soil-organic material (OM) mixtures were mixed with a 3D rotary shaker and conditioned at 15-20 % humidity, in relation to the water holding capacity of Ouroux and Dijon respectively. These mixtures were then incubated in closed plastic bins at room temperature for 180 days. Each bin contained 30 kg of an individually prepared mixture, the experiment being carried out in triplicates. Samples were taken after 14 and 180 days of incubation. Results after 14 days of incubation and 5% OM are presented. Similar results but with various extents were obtained after 180 days of incubation also and with higher rates of OM.

Soil suppressiveness to Fusarium wilt and R. solani *diseases*

The suppressiveness of natural soils, sterilized soils and soil-OM mixtures to Fusarium wilt of flax was determined using the bioassay proposed by Alabouvette *et al* (1982). Different soil mixtures (amended or unamended) were infested with a pathogenic strain of *F. oxysporum f. sp. lini* (Foln3) and the wilt incidence on a susceptible cultivar of flax (*Linum usitatissium* Opaline) was observed under controlled climatic conditions twice a week, after 3 weeks post inoculation, up to 8 weeks.

The suppressiveness of natural soils, sterilized soils and soil–OM mixtures to damping-off due to *R. solani* was assessed using the bioassay proposed by Camporota (1989). Ten day-old pine seedlings (*Pinus nigra* austrica) were inoculated with 30 g of the different soils and soil-OM mixtures inoculated with a pathogenic strain of *R. solani* AG2-2 (G6). The number of damped-off pine plantlets was noted after 3 days of incubation, twice a week up to 21 days. For the controls, flax plants and pine plantlets were grown with non-infested soils or soil-OM mixtures. The experiments were carried in greenhouse and each treatment was replicated three times. Randomized complete block designs were used. Survival data analyses, analysis of variance (ANOVA) and multiple comparison of means (PLSD Fisher's test, p=0.05) were performed to assess the effect of addition of compost or manure on soil suppressiveness to the diseases.

Soil microbial biomass

Soil microbial biomass was assessed by the technique described by Wu *et al.* (1990). Organic C originating from both fumigated and non fumigated soil or soil-OM mixture was extracted with 0.05 N K_2SO_4. Extractable organic C was measured with a Dohrmann DC80 analyzer. The results were expressed as mg biomass C kg^{-1} dry soil or soil-OM mixture. The means of three replicates per treatment were compared by ANOVA and PLSD Fisher's test ($p = 0.05$).

Community Level Physiological Profile (CLPP) of bacteria

Intensity and diversity of bacterial metabolism were evaluated by the technique proposed by Garland and Mills (1991) using Biolog GN microtiter plates. These 96 well microtiter plates contains 95 different carbon sources, a negative control and tetrazolium dye. Microorganisms were extracted from the uninoculated or inoculated soil treatments using a standard extraction method. Each well of the microtiter plate was inoculated with 150 µl of diluted soil suspension, containing 50 µg/ml cycloheximide solution to prevent fungal development. Plates were incubated at 25°C. Color formation was measured at 590 nm with a microtiter plate reader (Molecular Device). Readings were made at regular time intervals for 135 hours. Absorbance values for the wells with C sources were blanked against the control well. Average Well Color Development (AWCD) of each microtiter plate was calculated as the mean of the blanked absorbance values for all 95 response wells per reading time. The experiment was replicated thrice. The rate and the level of color development were evaluated and compared using ANOVA and Fisher's test. Community Level Physiological Profiles (CLPP) were determined at equivalent AWCD (0.4 OD units). Principal Component Analysis (PCA) was done to compare the samples. Subsequently, ANOVA was performed using the pair-wise Euclidean distances among replicates and treatments calculated from their coordinates on the five first axes.

Fungal community structure

A terminal restriction fragment length polymorphism (T-RFLP) fingerprinting method was used to compare the structure of fungal communities of unamended and amended soils (Edel-Hermann and Pérez-Piqueres, unpublished data). The DNA was extracted from 1g of soil (or soil-OM mixture) using a chemical extractant (sodium dodecyl sulfate) and a physical disruption (bead-beater). The DNA extracts was purified through spin columns first with polyvinylpolypyrrolidone (PVPP) to remove co-extracted humic acids, then with Sepharose 4B. DNA extractions were performed in triplicate for each soil and soil-OM mixture sample. The primers nu-SSU-0817-5' and nu-SSU-1536-3' (Borneman and Hartin 2000) were used for the direct amplification of fungal small subunit (SSU) rDNA from soil using a Polymerase Chain Reaction (PCR) procedure developed for subsequent T-RFLP analysis. The primer nu-SSU-1536-3' was labeled at its 5' end with the fluorescent dye D3 (Beckman Coulter, Fullerton, CA). Fifty ng of the purified PCR products were digested with the restriction endonucleases *Alu*I and *Mbo*I. The length polymorphism of the terminal restriction fragments generated by the restriction enzymes and labeled by the fluorescent dye was analyzed through capillary electrophoresis sequencer CEQ™ 2000XL (Beckman Coulter). The analysis provided fingerprints of the distribution and intensity of terminal restriction fragments found in the DNA extracts originating from the different soils and soil-OM mixtures. These fingerprints characterized the fungal community structures of the soils and the soil-OM mixtures. The relationship among the fingerprints were examined using correspondence analysis.

Results

Soil suppressiveness to diseases

To assess the nature of the mechanisms involved in soil suppressiveness to Fusarium wilt and Rhizoctonia diseases, bioassays were performed using both natural soils and sterile soils. Fusarium wilt suppression was very high in the natural soil of Dijon soil and less important in the natural soil of Ouroux (Figure 1).

However, when both soils were sterilized, they became conducive to the disease. A different scenario was observed when considering *R. solani* disease. The natural soil of Dijon was very conducive to the disease and became significantly less conducive once sterilized. The high level of suppressiveness to *R. solani* disease of the natural soil of Ouroux was not significantly altered by the soil sterilization.

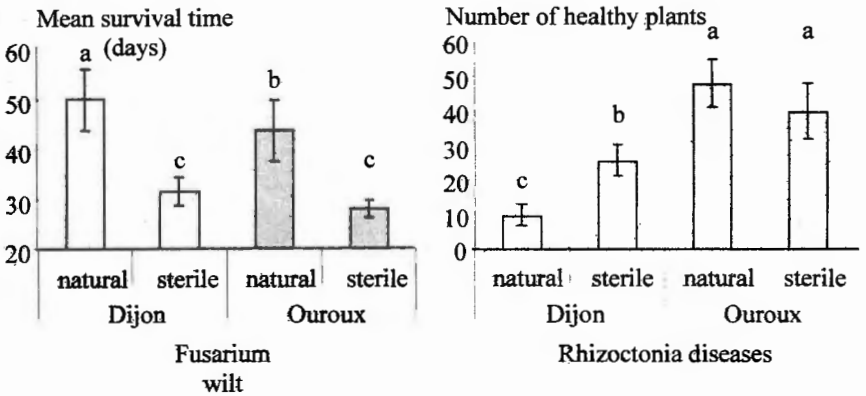

Figure 1. Assessment of soil suppressiveness to Fusarium wilt and to *R. solani* diseases in the natural soil and in the disinfected soil of Dijon and Ouroux. Different small letters on top of the bars indicate significant differences ($p<0.05$).

Bioassays were also performed to evaluate the consequences of organic amendments on the suppressiveness of the soils to the diseases. Soil suppressiveness to Fusarium wilt was not altered when compost or manure was brought to the suppressive soil of Dijon while the addition of OM to the moderately suppressive soil of Ouroux resulted in a significant increase in disease suppression (Figure 2).

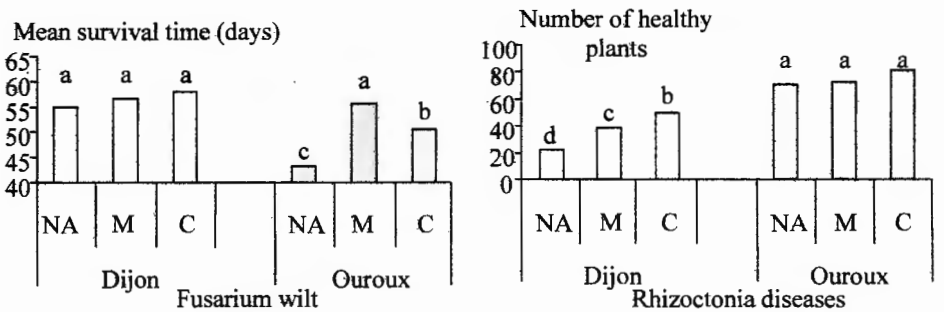

Figure 2. Assessment of soil suppressiveness to Fusarium wilt and *R. solani* diseases in non amended soil (NA) and in soil amended with 5% compost (C) or with 5% manure (M). Different small letters on top of the bars indicate significant differences ($p<0.05$).

Conversely, the organic amendments significantly increase soil suppressiveness to *R. solani* disease of the conducive soil of Dijon while they did not altered the already noticed high level of soil suppressiveness to the disease in the soil of Ouroux.

Structure and activity of the microbial communities

Significant changes in the soil biomass were only detected when more than 5% OM (v/v) were brought to the soil of Dijon. Increases were more pronounced with compost than with manure. In the soil of Ouroux, biomass was significantly increased when compost was brought to the soil, but to similar extents whatever the rate, while a low but significant increase in biomass was only detected when 30% (v/v) manure was brought to the soil.

CLPP were assessed at the bacterial community level in amended and non-amended soils. The progress curve of the AWCD during incubation time revealed that the rate of substrate utilization was significantly higher in the soil of Dijon (0.019 AWCD unit/h) than in the soil of Ouroux (0.008 AWCD unit/h) (Figure 3). As well the level of color development of the bacterial community (AWCD at the plateau) was significantly higher in the soil of Dijon (1.2 AWCD) than in the soil of Ouroux (0.65 AWCD).

The organic amendments did not significantly increase the substrate utilization rate of the bacterial community in Dijon but the addition of composts resulted in a significantly higher level of color development (1.4 AWCD) than in non amended soil or soil-manure mixture (1.3 AWCD). In the soil of Ouroux, the manure amendment and at a larger extent the compost amendment significantly increased both the rate of substrate utilization (0.018 and 0.04 AWCD unit/h respectively) and the level of color development of the bacterial community (1.05 AWCD and 1.3 AWCD respectively).

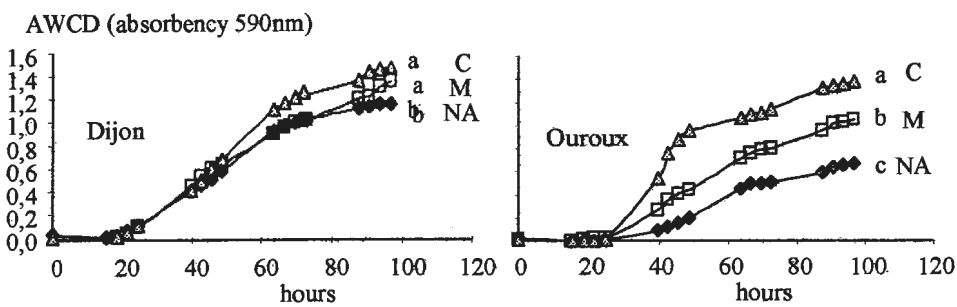

Figure 3. Average Well Color Development (AWCD) of bacterial communities originating from the non amended soil (NA) of Dijon and Ouroux, and in the same soils amended with 5% compost (C) or 5% manure (M). AWCD was assessed using Biolog GN microtiter plates. Different small letters at the right end of each kinetics indicate significant differences ($p<0.05$).

The CLPP established at AWCD = 0.4 were analyzed by comparing pair-wise Euclidean distances provided by the PCA between samples within and among treatments. The diversity of the metabolism achieved by the bacterial community of the soil of Dijon was significantly different from the one of the bacterial community of Ouroux. However, although the intensity

of the metabolism was enhanced in Ouroux soil and at a lesser extent in Dijon soil, the diversity remained unchanged in both soil-compost and soil-manure mixtures.

The structure of the fungal community of the soil of Dijon differed from that from the soil of Ouroux (Figure 4). In both soils, the addition of OM resulted in a shift in the original fungal community structure as illustrated in Figure 4 with the manure amended soils. The shifts in the fungal community structure caused by the addition of manure or compost were different in the two soils. Moreover manure and compost caused different shifts in the structure of the fungal community of the soil of Dijon or the soil of Ouroux.

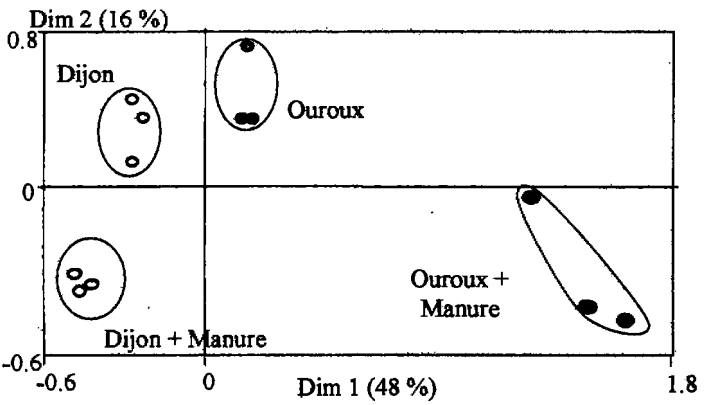

Figure 4. The structure of the fungal community in the soil of Dijon was different from that of the soil of Ouroux, as revealed by the correspondence analysis of T-RFLP fingerprints. Manure amendments provoked shifts in the structure of the fungal communities of both soils.

Discussion

The two soils were respectively conducive and suppressive to Fusarium wilt and vice versa to *R. solani* causing damping off. The pathogenic soilborne fungi *F. oxysporum* and *R. solani* have different ecological requirements leading to dissimilar reactions to the soil environment. The pathogenic activity of *F. oxysporum* was mainly limited by the microbial activity of the indigenous microflora in both soils while the abiotic traits existing in the sandy soil were restricting the development of the pathogenic activity of *R. solani*. However, amending these partially conducive soils with organic material enhanced significantly the degree of soil suppressiveness to a particular disease. These results were consistent with previous case-studies which already revealed the valuable disease suppression provided by the addition of composts to soil or potting mixture (Hoitink and Boehm 1999, Blok et al 2000, Cotxarrera et al 2002). Differential degrees of suppressiveness were observed with compost and manure against the two target pathogens and different mechanisms were revealed but in no case, the addition of organic amendments resulted in decreasing the original level of soil suppressiveness. In the soil of Ouroux, the soil environment remained unsuitable for the development of the pathogenic activity of *R. solani*.

In the soil of Dijon, the bacterial microflora was very active in utilizing nutrients provided by the microtiter plates. This revealed the competitive ability of this community

which therefore created a permanent oligotrophic situation in the soil and consequently prevented the germination and the pathogenic activity of *F. oxysporum*.

The addition of 5% compost or manure into the soil of Dijon did not significantly promote the biomass neither speed up the already active bacterial metabolism, but it is likely that the nutrients brought into the soil by this organic matter were efficiently used by the microflora. Consequently, the nutrients were no more available for *F. oxysporum*, hence the preservation of the degree of soil suppressiveness to Fusarium wilt. Surprisingly, *R. solani* was not affected by the bacterial metabolism in the soil of Dijon probably because, contrary to *F. oxysporum*, this fungal population is able to develop saprophytically independently from a minimal amount of available nutrients around the sclerotia (Deacon 1996, Steinberg et al 1999). Therefore, the enhancement of soil suppressiveness to *R. solani* diseases due to organic amendments should be related to the modification of the structure of the fungal community. Two compatible hypotheses might be envisaged : the organic amendments have stimulated the development of indigenous antagonistic populations or/and the organic amendments have brought in some fungal populations that developed in the soil-organic amendment mixtures and specifically antagonized *R. solani*.

In the soil of Ouroux, the weak metabolic activity of the bacteria was mainly responsible for the degree of conduciveness of the soil to Fusarium wilt. Therefore the stimulation of the bacterial metabolism due to organic amendments increased the competitive ability of the microflora, leading once again to the oligotrophic state similar to the one observed in Dijon. Consequently, soil suppressiveness to Fusarium wilt was enhanced. However, some specific fungal populations able to antagonize *F. oxysporum* or *R. solani* might also have been stimulated or/and carried into the soil through the organic amendments.

One soil may be suppressive to one disease and conducive to others. The complexity of soil suppressiveness to diseases is based on the various interactions between the pathogen and the saprophytic or antagonistic microflora on one hand and between soil abiotic factors and the microflora on the other hand. So far, all the interactions have not yet been elucidated. For instance, the identification of the microbial populations stimulated by organic amendments and the evaluation of their putative role in soil suppressiveness appeared as a promising challenge to understand some of the mechanisms involved in the limitation of the pathogenic activity of several pathogenic fungi in a soil. But also, this study revealed that disease suppression can be enhanced in soil by organic amendments, representing a long term potential management practice for Fusarium wilt and *R. solani* damping-off, when applied at conventional rates. This approach is consistent with the main objectives actually continued in the framework of EU-Projects (Compost Management 2001-2005, Recoveg 2001-2004). In this projects, the disease suppression abilities of many composts are being tested and the side-effects of their introduction to soils are evaluated.

Acknowledgements

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Multitrophic Interactions in Soil and Integrated Control
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Perspectives and challenges of breeding towards resistance to soil-borne pathogens – sugar beet as an example

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Abstract: *Rhizomania* and *Rhizoctonia* root rot represent sugar beet diseases, which are not or only difficult to control by chemicals. Breeding towards resistance to these pathogens bears the chance of a durable solution of the problem. While breeding of *Rhizomania* tolerant varieties has been succesfully developed during the last 30 years, *Rhizoctonia* tolerant varieties should be supplemented at present by additional means of control in agricultural practice.

Key words: soil-borne pathogens, breeding, BNYVV, *Rhizomania*, *Rhizoctonia solani*, integrated approach

Introduction

The soil-borne *Rhizomania* disease (BNYVV Beet Necrotic Yellow Vein Virus, transmitted by *Polymyxa betae*) is found in all major sugar beet growing areas and causes root yield losses as well as negative changes in quality components. A chemical control of *Rhizomania* is difficult and only partially effective (Asher 1993).

Rhizoctonia solani (mainly Late Root Rot) has become an increasing problem in sugar beet during the last decade. Efforts to control the fungal pathogen are undertaken by breeding for tolerance as well as suppressing the disease by various agricultural means.

Breeding strategies

Depending on the sources of a certain resistance, the number of genes involved and their inheritance, a breeding programm has to be set up. In the case of BNYVV, a single dominant gene was found to have a major effect on resistance (Lewellen et al. 1987). In this situation, only one parent in a hybrid variety has to carry the resistance gene.

In the case of *Rhizoctonia solani*, resistance seems to be inherited more quantitativly (Hecker & Ruppel 1975) and the protection is more effective against late root rot than against seedling damping-off. In this genetic situation, it might be necessary in the long run to introgress resistance in both hybrid parents to reach a higher level of resistance.

Next to *Rhizoctonia solani*, many more soil-borne fungi, i.e. *Aphanomyces cochlioides*, *Fusarium* spp. and *Phoma* are serious threats for the sugar beet crop.

Breeding towards resistance to the various root rot diseases is performed with different intensities. With increasing relevance in the agricultural practice these diseases are objectives of (cooperative) research, observation trials, intensive breeding programms or combinations of these activities.

Present situation and Outlook

Rhizomania disease

Within the past 20 years, sugar yield of tolerant varieties could be increased by 30 % (figure 1). Nowadays growing of tolerant sugar beet varieties is without risk for the farmer because the demand for high performance under diseased as well as under non-diseased field conditions is realized.

Today, PCR and RFLP techniques provide a successful tool for identification and incorporation of the Holly resistance (major) gene into actual breeding material.

So far there is no "immunity" to the pathogen: BNYVV can still propagate to some extent within the rootlets. Therefore present research is focussed on a further shift from tolerant to completely resistant varieties. A challenge for the future is to combine different sources of resistance in order to gain a higher level of resistance (major and minor genes) and to make this resistance environmentally more stable.

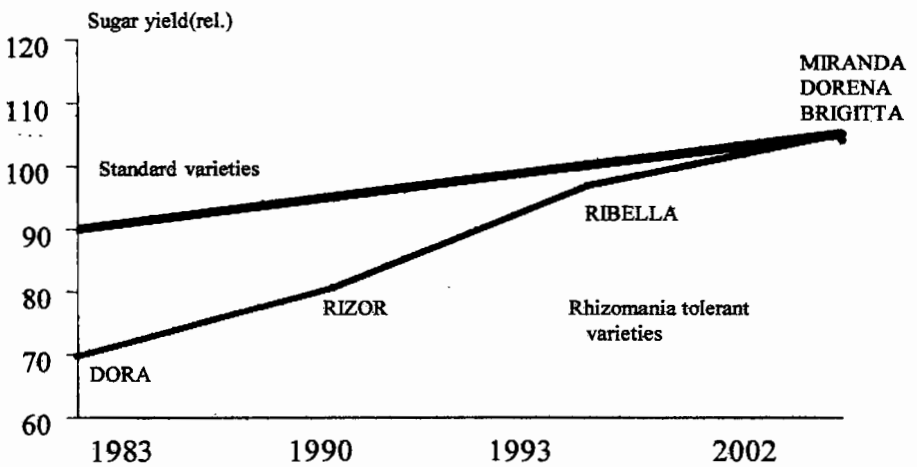

Figure 1. Sugar yield (rel.) of *Rhizomania* tolerant sugar beet varieties compared to sensitive varieties

Rhizoctonia Root rot

The present varieties with tolerance to *Rhizoctonia solani* are mainly derived from US breeding material (Panella & Ruppel, 1996). The breeding lines show a high level of resistance against Late Root Rot, but provide less protection in early plant stages. In contrast to BNYVV, resistance towards *Rhizoctonia solani* is based on more than one major gene (oligogene/multigene resistance).

Resistant lines bear additionally the disadvantages of poor seed quality (emergence), weak yield performance and low bolting resistance. Therefore the predominant target of breeding work is to overcome these negative features.

Additionally, current research in the seed technology department at KWS is focussed on the control of *Rhizoctonia solani* by means of seed treatments with special fungicides and/or antagonists in order to prevent and overcome an early infection of the plant (Figure 2).

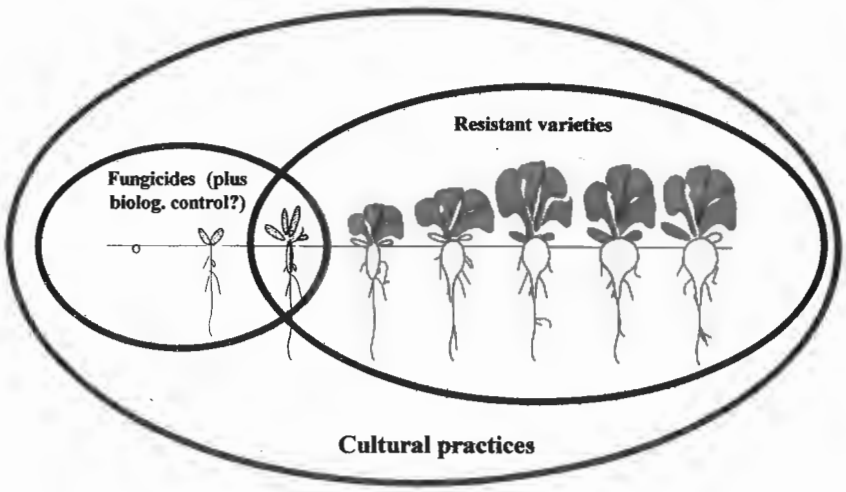

Figure 2. Concept of control of *Rhizoctonia solani* in sugar beet

In general the course of the disease and the extent of the damage are determined by the potential of infection in soil as well as by climatic conditions and cultivation methods (extension of crop rotation, improvement of soil structure).

To summarize, strategies of *Rhizoctonia solani* control have to be focused on an integrated approach including tolerant varieties, efficient seed treatments for protection in early plant stages and different means of cultural control in the rotation.

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Antifungal activity of a bacterium symbiotically associated with *Steinernema abbasi* towards *Fusarium oxysporum*

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Abstract: *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is the causal agent of the Fusarium wilt disease of tomato. Soil fumigant (mainly methyl bromide) applications are in use for its control. With the increasing environmental awareness, biological control methods are under investigation for their effectiveness, including the use of antagonists. *Pseudomonas oryzihabitans* (=*Flavimonas oryzihabitans*), a symbiont of the entomopathogenic nematode *Steinernema abbasi* was investigated as an antagonism of a *Fol* isolate in two laboratory and two glasshouse experiments. Bacteria and cell-free filtrate antifungal activity were tested both in dual cultures and in broth culture. In pot experiments, suspensions of bacteria in five concentrations (10^6, 10^5, 10^4, 10^3 and 10^2 cells/ml) were tested for their ability to control the pathogen at 25±3 ^{0}C. In all tests the bacterium significantly inhibited the growth of *Fol* mycelium *in vitro*. Similar results were obtained when the bacterium was also tested against *F. oxysporum* f. sp. *radicis lycopersici* and against *Rhizoctonia solani*. Moreover, when it was introduced into the soil, it was able to suppress the Fusarium wilt of tomato.

Key words: Biological control, Fusarium wilt, *Fusarium* oxysporum, *Pseudomonas*, *Flavimonas oryzihabitans*

Introduction

Insect pathogenic nematodes of the families Steinernematidae and Heterorhabditidae are symbiotically associated with bacteria of the genus *Xenorhabdus*. The nonfeeding, free-living infective stage nematode carries its bacteria symbiont monoxenically with the intestine. After entering an insect host, the nematodes invade the hemocoel and release its bacteria into the insect's haemolymph. The bacteria multiply, kill the insect, and establish suitable conditions for the nematodes reproduction by providing nutrients and inhibiting the growth of other micro-organisms (Akhurst 1983).

A new species of an entomopathogenic nematode *Steinernema abbasi* isolated from soil in Sultanate of Oman (Elawad *et al.*, 1999), has been shown to be a vector of the bacterium *Flavimonas oryzihabitans* (=*Pseudomonas oryzihabitans*) (Holmes *et al.*, 1987). This bacterium causes the *Galleria mellonella* larvae to die within 12 h. The bacterium activity results a rapid breakdown of the *G. mellonella* tissues on which the developing nematodes *S. abbasi* feeds.

P. oryzihabitans showed nematicidal properties *in vitro* against potato cyst nematodes (Andreoglou *et al.*, 2000), biological control of potato late blight (Elinor, 1999) and antifungal effects, due, mainly, to the production of specific compounds (Higuchi *et al.*, 1999). More over its strain INR-5, originally isolated from internal parts of cucumber roots, showed significant plant growth promotion induced systemic resistance activity under field trials (Wei *et al.*, 1996).

The purpose of this study was to investigate whether *P. oryzihabitans* could has any biological effect upon *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) *in vitro* or if the bacterium is able to induce soil suppressiveness to Fusarium wilt disease of tomato.

Material and methods

Microbial cultures

The pathogenic fungi used in this study were one isolate of *Fol* (IMI 194417), one isolate of *Rhizoctonia solani* provided by Dr. R.T.V. Fox (University of Reading) and one isolate of *Fusarium oxysporum* f. sp. *radicis lycopersici* (*Forl*) provided by Dr. Ioannou (Agriculture Research Institute Nicosia, Cyprus). The biocontrol agent studied was the bacterium *Pseudomonas oryzihabitans* (=*Flavimonas oryzihabitans*) isolated as a symbiont of the entomopathogenic nematode *Steinernema abbasi* (University of Reading). Fungi were maintained on Potato Dextrose Agar (PDA) (Difco) and the bacterium on Nutrient Agar (NA).

Preparation of inocula

Inocula of *Fol* were grown in 5% cornmeal-sand medium at 28 ^{0}C in the dark. Four weeks old culture of the pathogen was added to a sandy-loam (1:3, w/w) soil mixture in 5% (w/w) dilution. *P. oryzihabitans* bacterial cells from *S. abbasi* were isolated from the oozing haemolymph of infected wax moth *Galleria mellonella* larvae and plated onto nutrient agar, incubated at 28 ^{0}C in the dark for 24 h and then placed in a shaking incubator (150 rev/min) for 24h.

In Vitro Screening Fungal inhibition assay

The ability of *P. oryzihabitans* to inhibit growth of *Fol*, was determined in vitro on NA plates (25 ml per plate). Dual cultures were set up at 85 mm petri dishes, by placing discs, 5 mm in diameter, of the involved fungal isolates and at the opposite side, parallel streaks of *P. oryzihabitans*. Zones of colony growth inhibition were recorded 144 hours after inoculation, and they were estimated using the formula: [(R1-R2) / R2]* 100, where R1 = the largest and R2 = the smallest radius of the fungus. Each treatment was replicated 10 times.

Nutrient competition as a mechanism of more diffusible metabolites production

The production of anti-fungal bacterium diffusible metabolites against *Fol* was assessed in dual culture in a very low (4 g/L), low (14 g/L), normal (28 g/L) and high (37 g/L) NA concentration. Each treatment was replicated 10 times.

Effect of pre-incubation time

In dual cultures the bacterium *P. oryzihabitans* was incubated for longer period (48 h) compared to the 24 h incubation period to investigate where the bacterium cells are able to diffuse more inhibitory compounds against the tested fungi (*Fol, Forl, R. solani*). Each treatment was replicated 10 times.

Antifungal activity of cell-free culture filtrates

P. oryzihabitans cells were grown in 30 g/L Nutrient Broth No2 (NB OXOID Ltd.) for 24 h at 28^{0}C in a shaking incubator (150 rev/min) for 24 h. Bacteria cells were separated by centrifugation at 4100 g for 30 min. The supernatant was then sterilized by filtration through 0.22μm membrane filters (Millipore Ltd.). Cell-free culture filtrates were added to Czapek Dox Broth, which was inoculated with 3 plugs (3 mm in diameter) of the *Fol* isolate in

dilution up to 1000-fold (were 1000 fold: 0.1% free culture filtrates added into the tested broth) and placed in the shaking incubator for 12 days. After 3, 6 and 12 days of incubation, respectively, were assessed the fungus fresh mycelium weight, the mycelium dry weight and its sporulation. Each treatment was replicated three times.

In vivo glass house screening experiments assays, for the ability of the bacteria to suppress Fusarium wilt disease of tomato were conducted in plastic trays. Inoculum of the *Fol* pathogenic isolate was grown in 5% Cornmeal-Sand Medium and then added in a sandy loam (1:3 w/w) soil mixture. Bacterial cell suspension in five concentrations (i.e. 10^6, 10^5, 10^4, 10^3, 10^2 cells/ml) were prepared and tested for their ability to control the pathogen by applying 100 ml from each concentration onto soil surface. The same test was repeated in 1L plastic pots, used four bacterial cells concentrations (10^6, 10^5, 10^4, 10^3 cells/ml). Tomato plants (at 2-4 leaf stages) were transplanted into pots and water the same time with 20ml/pot per treatment with bacterium suspension.

Statistical analysis

Analyses were performed employing both the SPSS statistical programme and Microsoft Excel. ANOVA and multiple range tests were applied to test differences between treatments and identify statistical differences between means, respectively.

Results and discussion

In Vitro Screening

The results showed that bacteria cells produce freely diffusible compounds that are able to inhibit fungal growth (figure 1a). This is in agreement with Vagelas *et al.*, 2000 results that *P. oryzihabitans* produce unidentified compounds, which inhibit mycelial growth in vitro. As the medium concentration was increased from 4 g/L up to 37 g/L, more inhibition growth zones were observed (figure 1a). An explanation of these results could be that the more rich in nutrients medium exists, the more freely diffusible compounds inhibiting *Fol* hyphae are produced from *P. oryzihabitans*. Similar results were obtained when the pre-incubation time was increased from 24h to 48h (figure 1b for *Fol*). Same results were produced tested bacteria against *F. oxysporum* f. sp. *radicis lycopersici* (*Forl*) and against *Rhizoctonia solani* (*Rsolani*), (figure 1b).

P. oryzihabitans free-cell culture filtrates in dilutions up to 1000-fold (0.1% substance) in broth culture caused significant damage to *Fusarium* hyphae (not presented) and to conidia production (Figure 2).

In Vivo Glasshouse Screening Experiments

P. oryzihabitans cells suspension amendment into the soil, in all concentration tested, except 10^2 cells/ml, suppressed the Fusarium wilt disease of tomato, giving statistical differences in tomato stem length and root fresh weight, compared to plants, untreated and infected with *Fol*. Best results were obtained with the bacteria concentration of 10^4 cells/ml. This treatment was statistically not different in tomato stem length, stem fresh weight and root fresh weight compared to the untreated control plants (P=0.05). In all other concentration (i.e. 10^2, 10^3, 10^5 & 10^6 cells/ml the disease suppression was intermediate (Table 1).

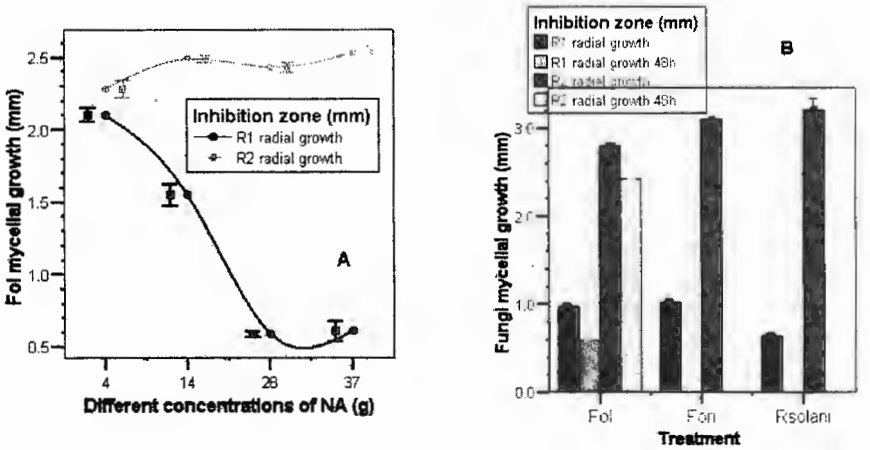

Figure 1. (a) Comparison of *P. oryzihabitans* anti-fungal activity in different NA concentrations; Differences among treatment determined using SPSS Drop lines graphs used error bars (Confidence interval 95%). Dots and lines are representing *F. oxysporum* f. sp. *lycopersici* mycelia growth. (b) Inhibition zone produced by the bacteria in different tested fungi used 24h and 48h pre-incubation time for bacteria. Bars show means; Error bars show mean ± SEM.

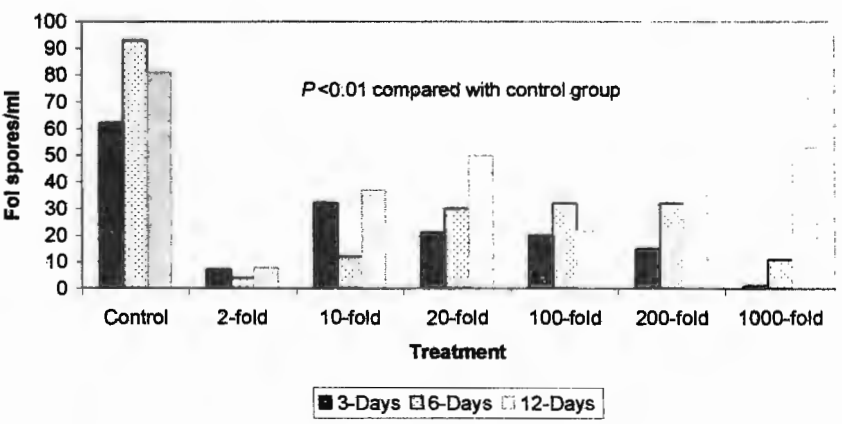

Figure 2. Inhibition of *F. oxysporum* f.sp. *lycopersici* spores in broth cultures amended with *P. oryzihabitans* cell-free filtrates.

Table 1. The effect of *P. oryzihabitans* cells soil amendment (suspension in different concentrations) on tomato stem length (mm), fresh stem weight (mg) and root weight (mg).

Treatment	Stem length	Stem fresh weight	Root fresh weight
Untreated	23.246 c*	128.719 d	41.711 f
F.o.lycopersici	10.035 a	17.698 a	11.147 ab
F.o.lycopersici / P. oryzihabitans 10^6cells/ml	18.129 bc	98.935 cd	18.120 bc
F.o.lycopersici / P. oryzihabitans 10^5cells/ml	21.770 bc	56.077 abc	22.236 bcd
F.o.lycopersici / P. oryzihabitans 10^4cells/ml	21.247 bc	121.648 d	40.350 f
F.o.lycopersici / P. oryzihabitans 10^3cells/ml	18.922 bc	102.484 cd	27.706 cde
F.o.lycopersici / P. oryzihabitans 10^2cells/ml	17.176 bc	31.077 a	16.547 ab

* Values within a column followed by the same later do not different significantly (P=0.05) according to Duncan's multiple range test. Values are based on 40 replicates per treatment.

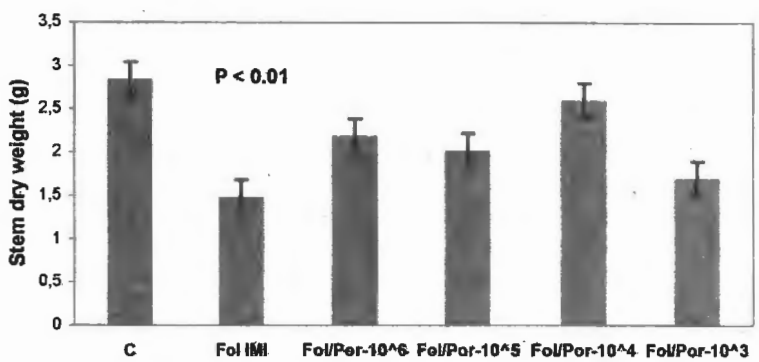

Figure 3. The effect of *P. oryzihabitans* cells soil amendment (suspension in different concentrations) on tomato stem dry weight (g) in pots experiment. Values are based on 15 replicates per treatment.

Same data were achieved testing the bacterium in pot experiments (Figure 3). In the same way as tray experiments a concentration of bacteria of 10^4 cells/ml resulted in a stem dry weight of tomato, significantly not different from the non infected control (Figure 3).

This study supports the report of Vagelas *et al.*, (2000) that the *P. oryzihabitans* entomopathogenic strain, produces substances toxic to *Fusarium oxysporum* f. sp. *lycopersici* the causal agent of tomato wilt disease. In this study it was proved that *P. oryzihabitans* not only inhibits *Fol in vitro*, but also suppresses Fusarium wilt of tomato, when it was added in soil. In this study it was proved that cell-free filtrates of the bacterium significantly inhibited the fungal sporogenesis.

The antifungal effect of the *P. oryzihabitans* strain, which was tested, is attributed to secondary diffusible metabolites. It will be worthwhile investigating whether these compounds are the same as those reported by Andreoglou *et al.*, (2000), that are produced by the same strain of *P. oryzihabitans* into agar medium and act directly against unhatched second stage juveniles (J_2s) of the potato cyst nematodes *Globodera rostochiensis.*

Chen et al., (1994), demonstrated that phase one bacteria from entomatogenic nematodes completely inhibited mycelial growth of *Botrytis cinerea, Ceratocystis ulmi* and *Pythium coloratum in vitro.* McInerney et al., (1991a; 1991b) reported that antibiotic compounds, named xenorhabdins and xenocoumacis 1 and 2 were isolated from cultures of *Xenorhabdus* spp. bacteria symbiotically associated with insect-pathogenic nematodes. These metabolites exhibit antimicrobial, insectidical, antibacterial, antifungal activities and potent antiulcer activity against stress-induced ulcers when dosed orally. Also, the entomopathogenic nematode *Sterinernema feltiae* and possibly its symbiotic bacteria have been shown to suppress damping-off caused by *Phoma betae* when nematodes die in the soil (Lopez-Robles *et al.*, 1997).

In conclusion, the present study showed that *P. oryzihabitans,* which is a symbiont to the entomopathogenic nematode *Steinernema abbasi,* produces diffusible metabolites, acting biologically against fungi and induce soil suppressiveness against Fusarium wilt of tomato.

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Soilborne fungi and bacteria symbiotically associated with *Steinernema* spp. acting as biological agents against Fusarium wilt of tomato

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Abstract: An isolate of *Gliocladium virens* from disease affected soil in a commercial tomato greenhouse proved highly antagonistic to *Fusarium oxysporum* f.sp. *lycopersici*, used together with an isolate of the nematophagus fungus *Verticillium chlamydosporium*. Significant disease control was obtained when young mycelial preparation (on a food- base culture) of the *G. virens* together with *V. chlamydosporium* was applied in potting medium. Similar results were observed when a *Trichoderma harzianum* isolate was treated in combination with the *V. chlamydosporium* isolate. Most promising, in terms of minimizing the Fusarium wilt of tomato incidence, was also the effect of the bacteria associated with entomopathogenic nematodes (*Steinernema* spp.), *Pseudomonas oryzihabitans* and *Xenorhabdus nematophilus*.

Key words: Biological control, Fusarium wilt, *Fusarium oxysporum*, *Pseudomonas oryzihabitans*, *Xenorhabdus nematophilus*, *Gliocladium virens*, *Trichoderma harzianum*, *Verticillium chlamydosporium*.

Introduction

Direct application of antagonists into the soil continues to be the principal method for biological control of soil-borne plant pathogens (Lewis & Papavizas, 1985). Population densities of isolates of *Trichoderma* Pers. Ex. Fr., *Gliocladium* Corda, *Aspergillus* Mich. Ex. Fr. and *Talaromyces* C. R. Benjamin, were showed to increase up to 10^6 –fold in natural soils amended with mycelial preparations of young cultures on a food-base (bran) rather with a conidial preparation (Lewis & Papavizas, 1984). Fermented biomass preparation of isolates of *Trichoderma* spp. and *Gliocladium virens*, abundant in chlamydospores, were more effective than conidia in reducing inoculum of *Rhizoctonia solani* (Beagle-Ristaino & Papavizas, 1985) in soil. It was also demonstrated that Fusarium wilt of tomato was significantly reduced by the amendment of a young mycelial preparation of *G. virens* and *V. chlamydosporium* isolates grown on corn-meal medium into the soil (Vagelas *et al.*, 2000a). Rhizobacteria, as *Flavimonas oryzihabitans* (=*Pseudomonas oryzihabitans*), strain INR-5 originally isolated from internal parts of cucumber, are referred as part growth-promoting rhizobacteria (PGPR) (Wei *et al.*, 1996). The INR-5 bacterial strain was shown to induce systemic resistance (ISR) by inhibiting the accumulation of the secondary plant metabolite cucurbitacin, causing a shift in the metabolic pathway to produce other anti-microbial compounds. Furthermore this bacterial strain may elicit the induction of other plant defence mechanisms (i.e., phytoalexin production and other compounds involved in ISR) against pathogens after it has been introduced into the plant (Zehnder *et al.*, 2001). The bacterium *P. oryzihabitans* which is a symbiont to the entomopathogenic nematode *Steinernema abbasi* has been shown to cause

significant inhibition of Fusarium wilt of tomato *in vitro* and also to affect the pathogen in soil. The reduction of Fusarium wilt severity was expressed by the increased tomato stem length, as well as the fresh and the dry weight of tomato plants (Vagelas *et al.*, 2000b). The entomopathogenic nematodes *S. feltiae* and possibly its symbiotic bacterium *Xenorhabdus bovienii* have been shown to suppress damping-off caused by *Phoma betae* when nematodes die in the soil (Lopez-Robles *et al.*, 1997). This study investigates the biological effect of soil fungal isolates and bacteria associated with entomopathogenic nematodes, acting alone or in combination, in suppressing the Fusarium wilt of tomato.

Materials and methods

Pathogen *Fusarium oxysporum* f. sp. *lycopersici* (IMI 194417) was isolated from tomato plants *Lycopersicum esculatum* c.v. Tiny Tim, showing symptoms of vascular wilt disease. The strain was kept on Potato Dextrose Agar (PDA).

Fungal antagonists

The *Gliocladium virens* isolate was derived from the collection of the Plant Protection Laboratory of the T.E.I. of Larissa, Greece and it was isolated from greenhouse soil in central Greece (Gravanis F.T. & Xifilidou S., 2000). The nematophagus fungus *Verticillium chlamydosporium* (Vc10) provided by Dr. B. R. Kerry IACR Rothamsted, and *Trichoderma harzianum* Rifai provided by CABI Bioscience, Egham, were used for the investigation. The strains were kept on PDA.

Entomopathogenic bacteria *P. oryzihabitans* and *X. nematophilus* were isolated from *Galleria mellonella* infected with nematodes using the method described by Akhurst (1983). Pure colonies were multiplied in 3% Nutrient Broth No2 (Oxoid), the suspension was centrifuge and the bacteria pellets were diluted with sterile tap water. Bacteria concentrations were determined using a spectrophotometer adjusted to the 600nm wavelength.

Fungi and bacteria on agar plates

The dual-culture technique was used (Royse & Ries, 1997), whereby mycelium plugs (4mm diameter) taken from PDA plates of *F. oxysporum* f.sp. *lycopersici* (*Fol*) and potential antagonist fungi, to be screened were placed 4 cm apart on PDA plates. In the case of the bacteria strains involved in this study, streaks were made at a distance of 3 cm from the *Fol* plugs. Antagonistic potentials (fungi and bacteria), quantified as percentage inhibition of growth, were evaluated after 144 h at 25°C, measurement of the radius of *Fol* colonies cultured with the potential antagonists relative to control plates with *Fol* alone using the formula: [100 - (R2 / R1) * 100), where R1 = the radius of the control colony in mm and R2 = the distance in mm travelled by the *Fol* colony over the tested antagonist's colony (Ahmed *et al.*, 1999). Mode of interactions were recorded as: 1, no inhibition; 2, mutual inhibition; 3, inhibition of *Fol* at distance; 4, inhibition of the pathogen by overgrowth; 5, inhibition of radial extension growth of *Fol* on initial contact between colonies; and 6, inhibition of potential antagonist by *Fol*. Dual cultures were tested on 3.7% PDA, 20% V-8 juice for fungi and 3.7%; 2.8% and 1.4% NA for the bacteria.

Inhibition action of antibiosis or toxins

Antibiotics or metabolites produced by fungal antagonists may inhibit the germination of the pathogen spores. To investigate whether the fungal antagonists employed in this study produced such antibiotics or metabolites in dual cultures, mycelium plugs (4 mm diameter) were placed 4 cm apart on PDA. Dual-cultures of *G. virens* and *V. chlamydosporium* were

incubated for seven days whereas dual-cultures of *T. harzianum* and *V. chlamydosporium* for four days. Ten single spores of Fol were placed in the inhibition zone of the two antagonists.

Fungi and bacteria in the field

The effect of the fungi and the bacteria on *Fol* were studied on 2 L pots containing a mixture of soil free of pathogens; peat: loam: sand (3:1:1, v:v). Pots were placed in the glasshouse and inoculated with 30 ml of a microconidial suspension of *Fol* in Czapek Dox broth (giving a final concentration of 4 x 10^4 microconidia per ml soil). Pots were left in the glasshouse for a month until *Fol* chlamydospores were formed (De Cal *et al.*, 1997). Tomato plants (at 2-4 leaf stages) were transplanted into pots and kept in the glasshouse at 25°C ± 5 for 60 days. *T. harzianum* and *G. virens* were grown in sand-bran (Lewis & Papavizas 1984) and corn meal-sand medium, respectively, in order to produce 10^3-10^4 young actively growing propagules per gram of soil. Sand-brand and cornmeal-sand medium were mixed with soil to provide 1% of mixed added medium. Seedlings were watered at the same time with the bacterial strains 20 ml/treatment (10^5 cells per ml/pot for *P. oryzihabitans* and *X. nematophilus*, respectively). *V. chlamydosporium* was grown in maize-sand medium for four weeks at 25°C in the dark. Fresh inocula were added into the potting soil mixture in a 2% dilution. Visual assessment of symptoms (disease severity %) arising from infection by *Fol* was made before the harvest, according to De Cal *et al.*, 1997).

Statistic analysis

Analyses were performed with the SPSS statistical programme. ANOVA was used to test differences among treatments prior to application of multiple comparison techniques. The least significant difference (LSD) was used to compare the treatment means.

Results and discussion

In dual-culture experiments *T. harzianum* and *G. virens* reduced radial growth of *Fol* from 85% to 88% on PDA and from 60% to 94% on V8Agar (Table 1). *G. virens* in both media overgrew *Fol* mycelium in 48 h, preventing its growth. The *Fol* hyphae, which were close to the *G. virens* hyphae, were white in colour and thicker than normal. Severe inhibition of the *Fol* mycelium growth occurred by abundant aerial *G. virens* mycelium when the two isolates were in contact. *T. harzianum* also overgrew the *Fusarium* mycelium in 48h. In both cases, *T. harzianum* overgrew the pathogen, and its hyphae coiled around the *Fol* hyphae causing their vacuolization and disintegration. *V. chlamydosporium* caused temporary inhibition zones on the *Fol* colony (Table 1). The *Fol* hyphae which were close to the *V. chlamydosporium* colony were white-pink in colour. *V. chlamydosporium* produced yellow rings when its hyphae were in contact with the *Fol* hyphae. Abundant chlamydospores were observed in these yellow rings *P. oryzihabitans* damaged the *Fol* hyphae probably by diffusible compounds it produced. More fungal inhibition was achieved in the high concentrations of NA. Less damage to *Fol* hyphae was caused by the bacterium *X. nematophilus* (Table 2).

Table 1. Mean % inhibition of radial extension growth of *F. oxysporum* after 144h by three potential antagonists in dual culture screen at 25°C

Antagonist	Mode[a]	Mean[b] % inhibition on PDA	Fusarium[c] pigmentation	Mean[b] % inhibition on V8A
V. chlamydosporium	2	14.4 ± 1.85	white-pink	Not tested
G. virens	5	85.4 ± 2.37	white	93.9 ± 0.91
T. harzianum	4	88.3 ± 3.98	cream	59.7 ± 1.53

[a] Mode of action of antagonist. [b] Mean % ± SEM. [c] *F. oxysporum* f. sp. *lycopersici* mycelial colonies appear on PDA; Values are based on 10 replicates per treatment.

Table 2. Mean % inhibition of radial extension growth of *F. oxysporum* after 144h by two potential antagonistic entomopathogenic bacteria in dual culture screen at 28°C.

Antagonist	Mode[a]	Mean[b] % inhibition on 37g NA	Mean[b] % inhibition on 28g NA	Mean[b] % inhibition on 14g NA
P. oryzihabitans	3	76 ± 2.28	76 ± 1.36	38 ± 1.06
X. nematophilus	3	28 ± 2.80	29 ± 1.67	15 ± 1.30

[a] Mode of action of antagonist. [b] Mean % ± SEM. Values are based on 10 replicates per treatment.

An intense inhibition zone was observed between the hyphae of the two challenged fungi. Metabolites produced by both *G. virens* and *V. chlamydosporium* inhibited the hyphal growth of the respective fungus when they were grown in dual-culture. The same results were observed when *T. harzianum* and *V. chlamydosporium* were grown in dual culture, but only for the first five to seven days of incubation. After that, *T. harzianum* overgrew the *V. chlamydosporium* hyphae. Within this zone, none of the *Fol* single spores (placed manually) were germinated. These results show that both isolates in both cases produced either antibiotics or other metabolites, which were deleterious to *F. oxysporum* spores.

All treatments significantly reduced tomato wilt disease and stem fresh weights compared to the infected controls (Table 3).

All treatments also, except *V.* chlamydosporium, significantly reduced stem length compared to the infected controls (Table 3).

Treatments with *G. virens, T. harzianum* and *P. oryzihabitans* had no significant difference with the untreated control (P=0.05), but were better in increasing plant fresh weight, compared to the effect of *V. chlamydosporium* and *X. nematophilus* were not significantly different from the untreated controls. Treatments with *G. virens* alone and in combination with *V. chlamydosporium* as well as *T. harzianum* in combination with *V. chlamydosporium* resulted even higher plant fresh weights, hence disease reduction. The last two treatments have given even better results than the untreated control, increasing plant fresh weight to 118-157% and 117-133%, respectively, over the controls. This increased growth response on tomato plants from *G. virens* alone and in combination with *V. chlamydosporium* treatments as well as from treatment with *T. harzianum* combined also with *V. chlamydosporium*, could be explained by the hypothesis that these organisms produce growth regulating metabolites. Vagelas *et al.*, (2000a) also demonstrated this hypothesis, when *G.*

virens, in combination with *V. chlamydosporium* responded in the same way, when applied on a food- base culture.

The results in this study support that the bacteria which were used, exhibit effects on tomato Fusarium wilt disease.

Table 3 Screening potential control agents *in vivo* tests against *F. oxysporum* f. sp. *lycopersici*.

Treatment	**Length (cm)**	**Fresh weight (g)**	**Disease severity[a] (%)**
Control (Untreated)	58.0 b*	63,9 bc	00 a
F. oxysporum f. sp. *lycopersici* (F.o.l)	46.5 a	48,0 a	56 e
V. chlamydosporium / F.o.l	49.5 a	58,3 b	42 d
G. virens / F.o.l	59.6 b	69,6 cd	24 b
T. harzianum / F.o.l	75.0 c	62,7 bc	33b cd
G. virens + Vc10 / F.o.l	60.7 b	75,5 d	23 b
T. harzianum + Vc10 / F.o.l	58.3 b	74,7 d	33b cd
P. oryzihabitans / F.o.l	57.4 b	63,6 bc	30 bc
X. nematophilus / F.o.l	55.6 b	60,6 b	37 cd

Values are based on 10 replicates per treatment. * Values within a column followed by the same later do not different significantly (P=0.05) according to Duncan's multiple range test.

[a]Disease severity was evaluated using a 1-5 scale: 1, (0-24%, healthy plants; 2, (25-49%) lower leaves yellow; 3, (50-74%) lower leaves dead and some upper leaves wilted; 4, (74-100%) lower leaves dead and all upper lives wilted; 5, (100%) dead plants.

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Biological control of *Rhizoctonia* solani damping-off with a bacterium symbiotically associated with *Steinernema abbasi*

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Abstract: *Rhizoctonia solani* is a causal agent of damping-off of may cultivated plants. An isolate of the bacterium *Pseudomonas oryzihabitans*, symbiotically associated with the entomopathogenic nematode *Steinernema abbasi*, strongly inhibited the pathogen *in vitro*. The bacterium was firmly attached onto fungus mycelia and degraded the cell walls of the pathogen. In greenhouse experiments, bacterial suspension in sterile water applied in the soil, effectively controlled damping-off of radish.

Key words: Biological control, Damping-off, *Rhizoctonia solani*, *Pseudomonas*, *Flavimonas oryzihabitans*

Introduction

Insect pathogenic nematodes of the family Steinernematidae are symbiotically associated with bacteria of the genus *Xenorhabdus*. The nonfeeding, free-living infective nematode stage carries its bacterial symbiont monoxenically with in the intestine. After entering an insect host, the nematodes invade the hemocoel and release bacteria into the insect's haemolymph. The bacteria multiply, kill the insect, and establish suitable conditions for the nematodes' reproduction by providing nutrients and inhibiting the growth of other microorganisms (Akhurst 1983).

A new species of an entomopathogenic nematode *Steinernema abbasi* isolated from soil in the Sultanate of Oman (Elawad *et al.*, 1999), has been shown to be a vector of the bacterium *Flavimonas oryzihabitans* (=*Pseudomonas oryzihabitans*) (Holmes *et al.*, 1987). This bacterium causes *Galleria mellonella* larvae to die within 12 h. The bacterium activity results in a rapid breakdown of the *G. mellonella* tissues on which the developing nematode *S. abbasi* feeds.

P. oryzihabitans showed nematicidal properties *in vitro* against potato cyst nematodes (Andreoglou *et al.*, 2000), biological control of potato late blight (Elinor, 1999) and antifungal effects, due, mainly, to the production of specific compounds (Higuchi *et al.*, 1999). Moreover its strain INR-5, originally isolated from internal parts of cucumber roots, showed significant plant growth promotion and induced systemic resistance under field trials (Wei *et al.*, 1996).

The purpose of this study was to investigate whether *P. oryzihabitans* could have any biological effect upon *Rhizoctonia solani in vitro* or if the bacterium is able to induce soil suppressiveness to *Rhizoctonia solani* damping-off disease of radish.

Material and methods

Microorganisms and culture conditions

A *P. oryzihabitans* strain symbiotically associated with *Steinernema abbasi* was cultured in media described by Andreoglou *et al.*, (2000). For in vitro studies and disease suppression tests, bacteria were grown and added to medium or soil as described by Vagelas *et al.*, (2000). An isolate of *R. solani* (obtained from Dr. R.T.V. Fox, University of Reading, U.K.) was cultured on PDA plates at 25 ^{0}C for 7 days. For the disease suppression assay, six 5-mm plugs of a *R. solani* were placed in a sterile Erlenmeyer flask containing 100g of autoclaved barley seeds with 25 ml of sterilized double-distilled water and incubated for 4 weeks at 25 ^{0}C. At the end of the incubation period the seeds, which were colonised by the *Rhizoctonia* mycelium, were reduced to small pieces, and using a sterile spatula, they were mixed. The resulting homogenous mixture was added to the soil.

Screening for enhanced inhibition of* R. solani *in vitro

Four concentrations (10^6, 10^5, 10^4 and 10^3 cells/ml) of the bacterium suspension were spread uniformly on the surface of Nutrient Agar (NA) in 9cm Petri dishes. At the same time plugs (4 mm in diameter) of the testing fungus, *R. solani*, were placed at the centre of the medium. Equal numbers of control plates were set up. Three concentrations, 1.4%, 2.8% and 3.7% of NA were tested. Replication was 10-fold.

Chemotaxis and lysis in vitro

In vitro chemotaxis and predator activity against fresh and freeze dry *R. solani* mycelial was assessed by inoculating soft (0.2%) agar plates centrally with a 50μl drop taken from a 24hours fresh broth of the bacterium (Issa and Wood, 1993). Then, resulting swarms were determined after 12 h of incubation at 28 ^{o}C. Replication was 10-fold.

Glasshouse experiment

Double-sterile sand-loam (1:3 v:v) was used. The soil was artificially inoculated with *R. solani* as described above mixed well in sterile bugs and placed in 1 L trays. Trays with infested moist soil were left for 5 days in the glasshouse at 28 ^{0}C and 100 seeds of radish were sown in each tray. At the same time bacteria cells were suspended in distilled tap water and the suspension was applied randomly in drops to cover the whole soil surface as described by Vagelas *et al.*, (2000). Four bacterium concentrations (10^6, 10^5, 10^4 and 10^3 cells/ml) were used. After 26 days, disease incidence was expressed either as percentage of diseased seedling or determined using the Sneh *et al.*, (1966) disease index (0-5). Fresh plant root and bulb weights were also recorded.

Statistical analysis

Analyses were performed employing the SPSS statistical programme. ANOVA and multiple range tests were applied to assess differences between treatments and identify statistical differences between means, respectively.

Results and discussion

Screening for enhanced inhibition of* R. solani *in vitro

When *R. solani* was grown on NA amended with *P. oryzihabitans* the fungus mycelium was significantly inhibited in 48 h (data not presented), in all bacteria concentrations (Table 1).

The inhibited mycelium was not colonized by *P. oryzihabitans*. The higher bacterial concentrations (10^5 and 10^6 cells/ml) effected stronger mycelium inhibition (Table 1).

Table 1. Effects of different bacterial treatments on inhibition of mycelia radial growth of *Rhizoctonia solani* on different concentrations of NA after 144h incubation at 28°C.

Treatment	Mycelia radial growth on 37g/L of NA	Mycelia radial growth on 28g/L of NA	Mycelia radial growth on 14g/L of NA
R. solani + *P. oryzihabitans* 10^6	0.0 a*	0.0 a	0.0 a
R. solani + *P. oryzihabitans* 10^5	0.0 a	0.2 a	0.8 a
R. solani + *P. oryzihabitans* 10^4	1.1 b	17.3 b	25.9 b
R. solani (Control)	10.9 c	18.8 c	27.1 b

*Values within a column followed by the same letter do not differ significantly (P=0.05) according to Duncan's multiple range test; Values are based on 10 replicates per treatment

Chemotaxis and lysis in vitro

P. oryzihabitans exhibited a significant chemotaxis response towards *R. solani* mycelia in soft (0.2%) agar compared with the control (Table 2). Also a significant response to detect prey from a distance was observed toward freeze-dried *R. solani* mycelium (Table 2). *P. oryzihabitans* exhibited predator activity against *Rhizoctonia* as the highly motile rod-shaped cells were observed microscopically to colonize the fungus mycelia plugs and degrade the mycelium cell walls. Some bacterial strains showed to exhibit chemotaxis towards nutrient-rich regions (Chet and Mitchell 1976), also Pseudomonas spp. exhibited chemotaxis in soil toward fungal exudates (Arora *et al.*, 1983). Similarly, strains of fluorescent *Pseudomonas* spp. exhibited chemotaxis toward soybean seed exudates in vitro and in soil (Scher *et al.*, 1985).

Table 2. Bacteria chemotaxis and lysis to *R. solani* mycelia *in vitro*

Treatment	Bacteria swarm radius growth (mm)	Characterization of swarm bacteria growth
P. oryzihabitans (control)	28.5 a*	Normal round growth
P. oryzihabitans with *R. solani* fresh mycelium	42.0 b	Strongly attracted and prey
P. oryzihabitans with *R. solani* freeze-dry mycelium	42.0 b	Strongly attracted and prey

*Values within a column followed by the same letter do not differ significantly (P=0.05) according to Duncan's multiple range test

Glasshouse experiment

Biological control of damping-off disease, caused by *R. solani*, in plants was achieved by applying cells of *P. oryzihabitans* to infested soil (Table 3). *P. oryzihabitans* applied to *R. solani*-infected soil at rates 10^6, 10^5, 10^4 and 10^3 cells/ml, delayed the appearance of symptoms and decreased diseased incidence from 73 to 24% (Table 3). Similarly, greater fresh root and bulb weights were achieved at rates of 10^6, 10^5, 10^4 and 10^3 cells/ml (Table 3). In this experiment *P. oryzihabitans* was similar survival in the soil in high and low application rates (Table 3). *P. fluorescens* induced soil suppressiveness to various soilborne fungal diseases and to produce several antibiotic metabolites (Maurhofer *et al.*, 1992). Some of the

latter have been identified. *P. oryzihabitans* showed nematicidal properties in vitro against potato cyst nematodes (Andreoglou *et al.*, 2000); of potato late blight (Elinor, 1999), of Fusarium oxysporum f. sp. lycopersici (Vagelas *et al.*, 2000), and also antifungal effects mainly due to the production of specific compounds (Higuchi *et al.*, 1999). More over its strain INR-5, originally isolated from internal parts of cucumber roots, showed significant plant growth promotion induced systemic resistance activity under field trials (Wei *et al.*, 1996).

Table 3. Biological control of *R. solani* with the bacteria symbiotically associated with *Steinernema abbasi* in soil

Treatment	Diseased plants (%)	Disease index[a]	Total root fresh weight (mg)	Fresh weight of healthy bulbs	*Pseudomonas oryzihabitans* Log_{10} (10^4 cfu/g of soil)
R. solani (Control)	73	3.65 b	439.6 a*	35.17 a	N
R. solani / *P. oryzihabitans* 10^6	29	1.92 a	894.4 ab	922.16 b	1.3591 b
R. solani / *P. oryzihabitans* 10^5	26	1.82 a	1357.4 b	1934.68 b	1.0962 a
R. solani / *P. oryzihabitans* 10^4	31	1.96 a	1205.7 b	1696.82 b	1.4772 b
R. solani / *P. oryzihabitans* 10^3	24	1.46 a	1009.5 b	1717.18 b	1.6230 b

[a]Disease index was evaluated using a 1-5 scale: 0 (healthy plants) to 5 (completely girdled); *Values within a column followed by the same letter do not differ significantly (P=0.05) according to Duncan's multiple range test

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Disease suppression in potting mixes amended with Dutch yard waste composts

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Abstract: Twelve yard waste composts were collected from different commercial composting sites in the Netherlands. Potting mixes were prepared consisting of 80% peat and 20% compost on a volume basis. Disease suppressiveness of the compost amended mixtures were compared with a nonamended mixture consisting of 100% peat in 3 bioassays: *Phytophthora cinnamomi* – lupin, *Rhizoctonia solani* AG 2-1 – cauliflower and *Cylindrocladium spathiphylli* – Spathiphyllum. None of the composts increased suppressiveness of the potting mixtures against lupin rot caused by *P. cinnamomi*. Nine and 3 composts increased suppressiveness in the *R. solani* and *C. spathiphylli* assay, respectively. Suppressiveness against *R. solani* incited damping-off decreased with increasing pH of the potting mixture.

Key words: *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Cylindrocladium spathiphylli*, Spathiphyllum

Introduction

Large amounts of peat are used in growing substrates in horticulture each year. In several European countries, the policy is to decrease the use of peat in growing substrates to preserve peat bogs as nature areas. Therefore, there is a need to look for alternative materials for potting mixtures which are now mainly composed of peat. Composts may replace part of the peat in potting mixtures and escpecially yard waste composts since they are relatively low in salinity as compared to other kinds of composts. Besides a lower use of peat, composted yard waste amendments may have the advantage of increasing the disease suppressive properties of the potting mixture due to an increase in general microbial activity and/or the presence of specific antagonists in the compost (Hoitink & Fahy, 1986; Hoitink & Boehm, 1999). However, not all composts increase suppressiveness of potting mixtures and, at the present, it is not possible to predict the suppressive effect of composted yard waste amendments. In general, compost characteristics which are easy to measure and which are predictive for the level of suppressiveness need to be known to make compost amendments a useful tool to control root pathogens in horticulture.

In January 2002, an EU-project has started on disease suppression induced by composts. Two of the main objectives of this EU-funded project, "Management of soil health in horticulture using composts" (compost management), are to find compost characteristics which are predictive for its suppressive properties and to find ways to render composts suppressive using selected antagonists. The present paper describes research on disease suppressive properties of potting mixtures amended with composted yard waste, which was carried out within the framework of the above mentioned EU-project. It describes results obtained with 12 Dutch yard waste composts and discusses the disease suppression in relation to the basal respiration of the

composts and the pH of the potting mixtures.

Materials and methods

Preparation of potting mixtures

Composts were collected and stored in loosely closed polyethylene bags at 4°C untill use. About 2-3 weeks before start of the bioassays a 1-L-sample of each compost was sent to a commercial laboratory (Groen Agro Control, Delft, the Netherlands) for determination of the pH and the mineral element contents in the 1:1.5 volume extract (Kipp *et al*, 2000). Eight days before start of the bioassays composts were incubated at room temperature and potting mixtures were prepared one day later. Nonamended mixtures consisted of peat (H2-3 on the Von Post decomposition scale), lime (8 g/L, Dolokal PG) and PG-mix (0.8 g/L, 15 – 10- 20). The same batch of peat was used in all tests. The peat (A0, Kekkila, Finland) was stored in compressed bales to which no lime nor mineral fertilizers had been added. Amended mixtures consisted of peat (80%) and compost (20%, volume basis) and mineral fertilizers. Lime (4 g/L) was added to 5 of the compost amended potting mixtures. Based on the mineral elements already present in the compost extract mineral fertilizers were added to the compost – peat mixtures to obtain similar nutrient levels in the nonamended and amended potting mixtures. Potting mixtures were incubated moist for one week at room temperature before start of the bioassays.

Bioassays

Disease suppressiveness of the potting mixtures was assessed in 3 different bioassays: *Phytophthora cinnamomi* – lupin, *Cylindrocladium spathiphylli* – Spathiphyllum and *Rhizoctonia solani* AG2-1 – cauliflower. Inoculum of *P. cinnamomi* (isolate kindly provided by Wim Blok, Wageningen University) and *C. spathiphylli* (isolate AN2000/11) were produced in mixtures of peat and ground oat as described by Blok *et al.* (2000). The inoculum was mixed with the potting mixture at a dosage of 0.1 % (volume basis). Pots with a diameter of 10.5 cm and a volume of 0.4 L were used in the *P. cinnamomi* assay. In each pot 8 lupin seeds (cv. Borweta) were sown and the number of healthy seedlings was determined 14 and 21 days later. Five non-infested and 5 infested pots were sown for each potting mixture. In the *C. spathiphylli* assay, young Spathiphyllum plantlets (cv. Ceres, Braam, De Kwakel, the Netherlands) were planted in pots with a diameter of 9 cm and a volume of 0.2 L . One replicate consisted of 7 pots placed in a tray (19 x 31 cm) on an irrigation mat. Each potting mixture had 5 replicates with infested soil and 3 replicates with noninfested soil. From 2 weeks after potting, plants were observed every 3-4 days and the number of healthy, diseased and completely wilted or dead plants noted. The final observation was carried out 7 weeks after potting. In the *Rhizoctonia* assay, cauliflower (Hybrid cv. Fremont) was sown in pots 9 cm in diameter with 9 seeds per pot. One week after sowing the number of seedlings in each pot was noted and a piece of agar (3 x 3 mm) with *R. solani* isolate 21R21 (obtained from Plant Research International, Wageningen, The Netherlands) was placed adjacent to one seedling near the edge of the pot. The agar-piece was cut from the margin of an actively growing colony on PDA and was placed just below the soil surface. Cauliflower seedlings were observed daily and as soon as all seedlings in one of the pots were damped-off the number of non-damped-off seedlings were determined in each pot. The *Phytophthora* and *Rhizoctonia* assays were carried out in a glasshouse at 20°C and the *Cylindrocladium* assay in a glasshouse at 24°C. Pots were placed on a dish or tray and irrigated regularly from below using tap water. Spathiphyllum plants obtained nutrient solution once a week from 4 weeks after

potting.

Suppressiveness of the different potting mixtures was determined in 3 series with 3, 5 and 4 composts respectively. In each series the disease levels were related to that of the nonamended control in order to compare suppression levels between the different series. The percentage disease suppression in the *Phytophthora* assay was calculated as:

$$100 - 100 \times (CM - IM)/(CM - IC),$$

where: CM is the number of healthy plants in the noninfested mixture, IM is the number of plants in the infested mixture and IC is the number of healthy plants in the infested unamended potting mixture.

In the *Cylindrocladium* assay the area under the disease progress curve (AUDPC) was calculated for the number of completely wilted or dead plants and the percentage disease suppression was calculated as:

$$100 - 100 \times (AUDPC_{\text{amended potting mixture}}/AUDPC_{\text{nonamended potting mixture}})$$

For the *Rhizoctonia* assay, %disease suppression induced by the compost was calculated as:

100 – 100 x (% damping-off in amended mixture)/(% damping-off in nonamended mixture).

Characterisation of compost and potting mixtures and statistical analysis

Basal respiration of the composts was determined using an automated system as described by Blok *et al.* (2000). One week after preparation of the potting mixtures, the mineral element contents, EC and pH of water extracts (1:1.5 volume) of the mixtures were determined by the commercial laboratory mentioned above. In each series of tests suppressiveness of compost amended potting mixtures were compared with the nonamended mixture using Fisher's Protected LSD ($P<0.05$). The relationship between disease suppression and basal respiration of the composts and the pH of the potting mixtures was investigated using simple linear regression analysis (GenStat for Windows 6th edition).

Results and discussion

The contents of mineral elements in the water extract of the various potting mixtures did not vary greatly (Table 1). The electrical conductivity (EC) of the water extract (1:1.5 volume) of the amended mixtures was higher than that of the nonamended mixtures but all EC-values were still acceptable for growing moderately salt sensitive plants (Kipp *et al*, 2000). The pH of the amended potting mixtures to which no lime had been added was below 5.0. Addition of lime (4g/L potting mixture) resulted in a pH of 5.5 –6.0 which is within the range recommended for most potted plants (Straver *et al*, 1999).

None of the compost amended potting mixtures significantly suppressed the *Phytophthora* disease in lupin as compared to the nonamended control. Basal respiration of the tested composts ranged from 9-19 μg CO_2/ml/h. Blok *et al.* (2002) found composts which significantly suppressed the disease among 9 Dutch biowaste composts tested at 2 different ages.

Disease suppression was highest using young composts and was related to the basal respiration of the composts (R^2 = 61%) which ranged from 3 – 42 μg CO_2/ml/h. Possibly, the composts tested in the present study were too stable to induce suppression against the

Phytophthora disease as the basal respiration of the composts was clearly lower than those of the composts inducing suppression in the study of Blok *et al.* (2000).

Table 1. Concentration of macro-elements in water extracts (1:1.5 volume extract) of nonamended and compost amended potting mixtures.

Elements	nonamended[x]		compost amended[y]	
(mmol/L)	min.	max.	min.	max.
pH	5.5	5.9	4.2	5.8
EC (mS/cm)	0.66	0.73	0.77	1.17
NH_4	1.4	1.7	0.6	1.7
K	1.0	1.3	2.7	4.4
Na	0.3	0.4	0.6	1.4
Ca	0.9	1.0	0.6	1.5
Mg	0.8	1.0	0.4	0.8
Si	0.3	0.4	0.3	0.7
NO_3	3.0	3.2	1.7	4.0
Cl	0.3	0.6	1.3	2.8
SO_4	0.6	0.8	0.3	1.4
HCO_3	0.2	0.2	0.0	0.2
P	0.5	0.7	0.7	2.0

[x] Minimum (min) and maximum (max) values of 3 nonamended potting mixtures
[y] Minimum and maximum values of 12 compost amended potting mixtures

Three and 9 composts increased suppressiveness of the potting mixtures against the *Cylindrocladium* and *Rhizoctonia* disease, respectively. Disease suppression against *Rhizoctonia* damping-off was negatively correlated with the pH of the compost amended potting mixture ($P<0.05$, $R^2 = 56\%$). A low pH may have a direct negative effect on *R. solani* but may also stimulate microbial antagonists present in the compost. A similar effect of the pH on the suppressive effect of a soil amendment was found by Huang and Kuhlman (1991). They found higher densities of *Trichoderma* spp. and *Penicillium* spp. with decreasing pH between pH 4 and 6 and concluded that the suppressive effect of the amendment at low pH was especially due to the colonization of the soil mixture by these micro-organisms. The recommended pH for potted plants is generally between 5.0 and 6.0. However, a pH of 5.0 will not negatively affect the growth of most plants and the pH may even be below 5.0 for some plants. So, amendment of yard waste compost in combination with a (moderately) low pH of the potting mixture may be a practicable tool for partial control of *Rhizoctonia* diseases in container grown plants tolerating such pH-values.

The work presented in the present paper is part of a larger experiment performed within the framework of the earlier mentioned EU-project "Compost Management" (http//www.ppo.dlo.nl/compost). In the larger experiment different types of composts are being tested to induce suppressiveness in soil and potting mixtures. Composts and compost amended substratres are being characterized biologically and physico-chemically by different research partners to identify compost characteristics by which the suppressive properties of composts may be predicted.

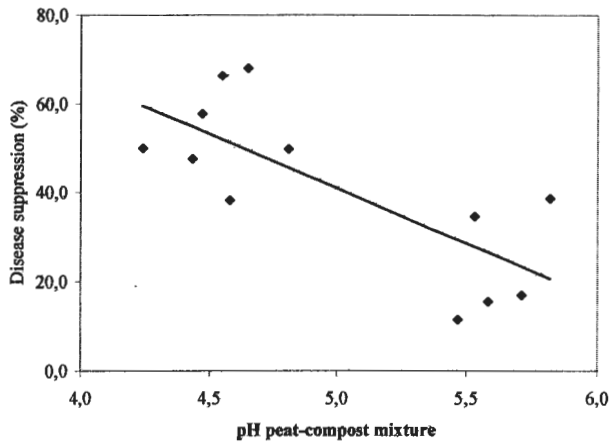

Figure 1. Relation between pH of yard waste compost amended potting mixtures and suppression of damping-off of cauliflower seedlings caused by *Rhizoctonia solani* AG2-1.

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Impact of application of biocontrol agents to plant root on the natural occurring microbial community

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Abstract: In three field experiments the impact of application of Biological Control Agents (BCAs) on the rhizosphere microbial community of the two *Verticillium* host plants strawberry (*Fragaria x ananassa* Duch.) and potato (*Solanum tuberosum* L.) was assessed. Two antifungal bacterial strains, one Gram-negative (HRO-C48, *Serratia plymuthica*) and one Gram-positive strain (HRO-7W1, *Streptomyces* spec.), were applied. Rifampicin resistant mutants of HRO-C48 established well in the rhizosphere of both plants. They were isolated even three months after application in abundances of about 10^3 to 10^4 CFU x g^{-1} root fresh weight. Concerning the abundances of culturable bacteria (CFU) in the rhizosphere and the proportion of *Verticillium* antagonists no differences between treatments and control plants were observed. Differences were obtained in the quality of species composition and diversity of *Verticillium* antagonists. By BOX-PCR fingerprints and fatty acid methyl ester (FAME) analysis of the antagonistic isolates a reduced diversity was found in the rhizosphere of *Serratia*-treated strawberry plants at the beginning of the vegetation period. In the rhizosphere of potato plants this effect could not be observed. As a cultivation-independent method to determine the influence of bacterial communities the single strand conformation polymorphism (SSCP) analysis based on the 16S rRNA genes was applied. Using general eubacterial primers, no difference in the bacterial community between *Serratia*-treated and non-treated plants was observed. Strain HRO-7W1 did not show any effects on the microbial rhizosphere populations in field.

Key words: risk assessment, SSCP, *Serratia*, *Streptomyces*

Introduction

Biological control using microorganisms to suppress phytopathogenic fungi is interesting as an alternative to the use of pesticides (Weller 1988, Bloemberg and Lugtenberg 2001). Because of the natural origin of biological control agents (BCAs), the advantage of biological control is a low or no toxicity for the environment, human health, and plants. The soilborne fungus *Verticillium dahliae* Kleb., the agent of Verticillium wilt, causes dramatic yield loss in many crops (Tjamos et al. 2000). The pathogen occurs worldwide and has a broad host range, e.g. strawberry, potato, and oilseed rape. Microsclerotia of *V. dahliae* may persist in soil and senescing tissues for several years in the absence of a susceptible host. Chemical control is nearly impossible. After the phasing out of methyl bromide as fungicidal substance efficacious control methods are urgently needed for commercial crop production. Antifungal bacteria occurring in the rhizosphere have been a focus of research in the field of soil-borne pathogens. Numerous studies demonstrated the ability of rhizobacteria to suppress diseases caused by fungal plant pathogens (Emmert and Handelsman 1999, Whipps 1997). Recently we reported the selection of rhizobacteria antagonistic to Verticillium wilt (Berg et al. 2000, 2001, Kurze et al. 2001).

As a consequence, the impact of applied microorganisms on the natural occurring root-associated community should be assessed. The bacterial rhizosphere communities of two *Verticillium* host plants, field-grown strawberry (*Fragaria X ananassa* Duch.) and potato (*Solanum tuberosum* L.), were investigated. Two *Verticillium* antagonists served as model organisms, the Gram-negative strain *Serratia plymuthica* HRO-C48 and the Gram-positive strain *Streptomyces* spec. HRO-7W1. This is one of the first projects dealing with risk assessment of biological control organisms relating to natural occurring bacteria.

Material and Methods

Biological control organisms

The strain *Serratia plymuthica* HRO-C48 was isolated from the rhizosphere of oilseed rape (Kalbe et al. 1996). It is a Gram-negative enterobacterial strain with high antagonistic potential against the phytopathogenic fungus *Verticillium dahliae* Kleb. due to the production of chitinases (Frankowski et al. 1998). The Gram-positive strain *Streptomyces* spec. HRO-7W1 originated from the rhizosphere of strawberry (Berg et al. 2000). It shows antifungal cell wall-degrading activity and produces siderophores and antibiotics (Berg and Lüth 1999, Berg et al. 2000). The strain HRO-C48 was fermented in sterile nutrient broth, the strain HRO-7W1 in DSM 65 medium. After one day of pre-incubation the BCAs were inoculated in a 10 liter fermenter (Biostat B, B. Braun Biotech International, Germany) and were incubated at 30°C and 150 rpm for two (HRO-C48) and four (HRO-7W1) days.

Plants and field experiments

The bacterial rhizosphere communities of two *Verticillium* host plants, field-grown strawberry and potato were investigated. Frigo plants of strawberry were planted on fields near Rostock/Germany and Münster/Germany in series with five replicates and about 50 plants per replicate. The potatoes were deposited on a field near Rostock/Germany in series with five replicates and 15 potato clods per replicate. The plant roots and pre-germinated potato clods were dipped into the bacterial suspensions HRO-C48, HRO 7W1 and water (control) for 15 to 20 min prior planting. In the field experiment near Rostock (strawberry) the wild type of strain HRO-C48, and in the experiments near Münster (strawberry) and Rostock (potato) the rifampicin resistant mutant of HRO-C48 were applied, and the wild type of strain HRO-7W1. In Münster strain HRO-C48 and a combination of strains HRO-C48 and HRO-7W1 were introduced into strawberry roots. The application concentration of HRO-C48 was about 10^9 CFU x ml^{-1}, and for HRO-7W1 10^8 CFU x ml^{-1}. Rhizosphere samples of 5 g of roots from three plants for each of five replicates were taken three times over one growing season.

Abundances of bacteria and rifampicin resistant mutants

The samples were pre-treated in Stomacher bags (Interscience, UK) in a bag mixer (model W, Interscience, UK) for 60 s. Determination of colony forming units (CFU) was performed by plating on R2A agar containing 100 ppm nystatin. To determine rifampicin resistant mutants of HRO-C48 nutrient agar containing 100 ppm rifampicin was used. Plates were incubated for three days at 20°C.

Proportion of antifungal isolates

For each of the three treatments in one experiment about 150 randomly chosen colonies (about 30 colonies per replicate) were transferred on R2A to screen for antifungal *in vitro* antagonism towards *Verticillium dahliae* Kleb. in dual culture assay. The isolated strains were used in an initial screening by dual testing on Waksman agar in 24-well microtiter plates. 20

µl of the *Verticillium* suspension (Czapek Dox medium (Difco), 20°C, 130 rpm) were dropped into each well. Some material was picked from bacterial strains and transferred to the fungus. The plates were incubated at 20°C for five days. The formation of an inhibition zone of fungal growth by the activity of the bacterium indicates an antagonistic strain. Antagonistic strains were tested once again on Petri dishes to measure the inhibition zones according to Berg et al. (2001).

Cluster analysis of the antagonistic strains by BOX-PCR fingerprints

The *Verticillium* antagonists were characterised by BOX-PCR fingerprints (Rademaker and De Bruijn 1997), identified by fatty acid methyl ester (FAME) profiles (Sasser 1990), and partly sequenced 16S r-RNA gene followed by an alignment with reference sequences using the BLAST algorithm (Altschul et al. 1997). An evaluation of the BOX-PCR fingerprints was made using GelCompare software (version 4.1, Applied Math, Kortrijk, Belgium). Cluster analysis was performed with the unweighted pair-group method with arithmetic mean (UPGMA).

Single strand conformation polymorphism (SSCP)

The SSCP analysis according to Tebbe et al. (2001) was used to analyse the structure of the rhizosphere community. Previously, a microorganism pellet was obtained by centrifugation and washing the samples in NaCl solution (0.85%). The polyacrylamidegel (7%) electrophoresis was performed on a DGGE/TGGE apparatus (Biometra, Göttingen, Germany) for about 19 hours at 26°C and 400 V.

Results and discussion

Impact assessment by cultivation-dependent analysis

The CFU numbers determined for the rhizosphere of strawberry plants ranged from $\log_{10}$ 8.2 to 7.1 CFU g^{-1} root fresh weight in both treatments and controls. No statistically significant differences (t-test) were found for both plants. The rifampicin resistant mutants of strain HRO-C48 were re-isolated in abundances of $\log_{10}$ 6.1 to 4.4 CFU g^{-1} root fresh weight with a seasonal decrease over the vegetation period in the rhizosphere of strawberry. In the rhizosphere of potato lower re-isolation rates were obtained with $\log_{10}$ 4.8 to 2.3 CFU g^{-1} root fresh weight. The applied strain could be found in the rhizospheres over three months, indicating a good establishment of the strain in both plants.

The proportion of isolates with antagonistic activity against *Verticillium* was higher for the strawberry rhizosphere (13.6%) than for the potato rhizosphere (6.1%). The numbers of antagonists in strawberry roots varied strongly between 3.1 and 47.4% in the treatments and between 3.6 and 52.5% in the control plants. In the rhizosphere of potato plants the proportion of antagonists was lower with 1.4 to 12.6% in the treated plant roots, and 2.8 to 8.1% in the untreated roots. The differences between the treatments and the control were not statistically significant due to a high variation (about 68%). Seasonal shifts could not be observed. A higher antagonistic potential of strawberry rhizosphere in comparison to oilseed rape and potato was recently described Berg et al. (2002).

PCR fingerprinting based on repetitive BOX elements in the bacterial genome (Martin et al. 1992) is a good method for the characterisation of single strains thanks to its simplicity and applicability to a variety of bacterial groups. Cluster analysis by UPGMA showed a reduced diversity of the antagonistic isolates in the rhizosphere of *Serratia*-treated strawberry plants at the beginning of the vegetation period. This result was confirmed by FAME analysis and 16S rDNA sequencing in two field experiments. Antagonistic isolates were mainly identified as

Serratia-strains. Similar results were obtained in the second sampling time, while the diversity became higher in the third. In the rhizosphere of potato plants no differences were observed between the treatments and sampling times.

To illustrate this result the general diversity of the bacterial communities was determined by the Shannon information theory function (Shannon and Weaver 1949) at an 80% similarity according the BOX-PCR fingerprint clusters accepting that all strains with higher similarity represent one genotype (Fig. 1).

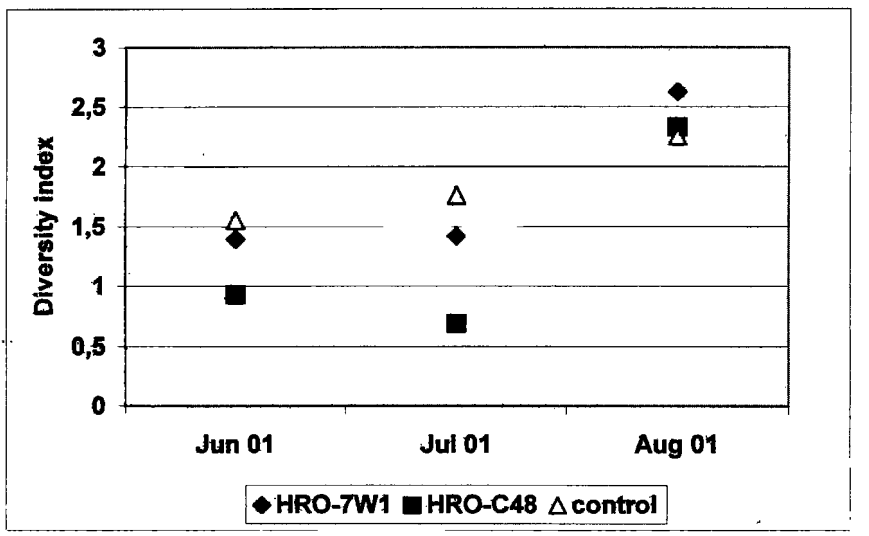

Figure 1: Diversity shifts of bacterial antagonists based on BOX-PCR fingerprint clusters at 80% similarity in the rhizosphere of *Streptomyces*-treated (HRO-7W1), *Serratia*-treated (HRO-C48), and control plants (strawberry, Rostock).

Impact assessment by cultivation-independent analysis

An analysis of the community structure by cultivation-independent methods is urgently needed for an impact assessment because only a limited knowledge of naturally occurring microbial diversity is offered by cultivation approaches. Various methods are accepted: temperature/denaturing gradient gel electrophoresis (T/DGGE), terminal restriction fragment length polymorphism (tRFLP), and SSCP.

As a cultivation-independent method the SSCP analysis based on the 16S rRNA genes was applied. Using universal eubacterial primers, no difference in the bacterial community between treated and non-treated strawberry and potato plants was observed. The patterns of the community seemed to be not influenced by an application, rather than by seasonal shifts. The dominance of strain HRO-C48 in these treatments was confirmed. Cloning and sequencing of the band corresponding to the band of the reference strain HRO-C48 revealed a similarity of about 93% to strain *Serratia plymuthica* DSM 4540. A band corresponding to the *Streptomyces* sp. strain HRO-7W1 could not be detected.

Conclusions

In Table 1 the effects from an application of biological control agents on the natural occurring bacterial community in the rhizosphere of strawberry are summarised.

Table 1: Summary of the impact of *Streptomyces* treatment (HRO-7W1) and *Serratia* treatment (HRO-C48) on the naturally occurring bacteria in strawberry rhizosphere

Parameters	**7W1**	**C48**	**control**
Abundances of bacteria	-	-	-
Establishment of the rifampicin resistant mutants		+	
Proportion of *Verticillium* antagonists	-	-	-
Diversity of *Verticillium* antagonists based on BOX-PCR fingerprint clusters	-	+	-
Gel patterns obtained by SSCP analysis of the 16S rRNA gene	-	+	-

-: no influence; +: influence

No effects on the abundances of bacteria and the proportion of *Verticillium* antagonists were observed. The main influence was to be found for the diversity of the antagonists. In the strawberry rhizosphere the applied strain HRO-C48 was dominant until the second sampling and reduced the diversity of *Verticillium* antagonists in this treatment. A cultivation-independent characterisation of the community by SSCP analysis of the 16S rRNA gene showed no influence on the other members of the bacterial community. An analysis of the community by group-specific bacterial and fungal primers needs to be completed. Changes in the species composition and diversity of root-associated bacteria due to factors like the plants themselves (root exudation, age), other microorganisms (competition, portion of pathogens), soil type and agricultural practice seems to be stronger than an application. Berg et al. (2002) demonstrated a strong plant dependence of the occurrence of *Verticillium* antagonists.

The aim of this work is the development of a model system to estimate the impact of an application of biocontrol agents (bacterial antagonists) on the rhizosphere communities. The chosen BCA HRO-C48 proved to be a good indicator due to an excellent root competence. For an impact assessment of the bacterial communities a combination of cultivation-dependent and cultivation-independent methods seems to be useful.

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